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(54) Title: HUMAN TIMP-3

(57) Abstract

The present invention provides isolated human TIMP-3. The polypeptide, specific antibodies prepared against it, and pharmaceutical compositions comprising such polypeptides and antibodies are useful, for example, in diagnostics, therapeutics, and prophylaxis of conditions characterized by excess or undesired activity of a matrix metalloproteinase.

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HUMAN TIMP-3

Technical Field

This invention is related to the field of protein biochemistry. Specifically, the invention relates to human tissue inhibitor of metalloproteinases (human TIMP-3).

Cross-References to Other Applications

This is a continuation-in-part of U.S. Application Serial No. 08/167,463 filed on December 12, 1993, pending, which is a continuation-in-part of U.S. Application Serial No. 08/105,263 filed on August 12, 1993, pending.

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Background Art

Matrix metalloproteinases (MMPs) are a family of enzymes that includes interstitial collagenase (MMP-1), 72-kDa and 92-kDa gelatinases (MMP-2 and MMP-9), stromelysin (MMP-3) and matrilysin (MMP-7). These enzymes are secreted by cells within tissues and by infiltrating inflammatory cells. Collectively, they are capable of degrading most of the proteins in the ECM.

MMPs display different substrate specificities yet have several properties in common. They are all zinc-containing enzymes that require calcium for activity. They are secreted as zymogens and activated in situ, usually by release of an inhibitory N-terminal pro-piece containing a single cysteine residue. The attached pro-piece is believed to coordinate with the zinc in the active site of the proteinase, thereby suppressing the proteolytic activity. Activation may be accompanied by additional proteolytic cleavages that can generate active enzymes of lower molecular weights.

All members of the MMP family have a short conserved region consisting of the HEXGH motif that provides two Zn-coordinating histidine residues and a glutamic acid residue that is considered part of the catalytic site.

The MMPs are inhibited by members of the family of tissue inhibitors of metalloproteinases (TIMPs, e.g., TIMP-1, TIMP-2, and TIMP-3), which bind at the active site and block access to substrate. Matrix remodelling, which occurs during various normal and pathological processes, depends upon a critical balance between activated MMPs inhibiting TIMPs. For reviews of the matrix metalloproteinases and their inhibitors see Alexander and Werb (1991), In: Cell Biology of Extracellular Matrix, ed. Hay, Plenum Press, New York, pp. 255-302; Murphy et al. (1991) Br. J. Rheumatol. 30:25-31; Woessner (1991) FASEB J. 5:2145-2154; Matrisian (1992) Bioessays 14:455-463; Birkedal-Hansen et al. (1993) Crit. Rev. Oral Biol. and Med. 4:197-250; and Denhardt et al. (1993) J. Pharmacol. Ther. 59:329-341.

TIMP-1 and TIMP-2. The tissue inhibitors of matrix metalloproteinases (TIMP) also comprise a family of proteins that includes TIMP-1, TIMP-2 and TIMP-3.

TIMP-1 is a glycoprotein that was originally characterized as a mammalian collagenase inhibitor with a molecular mass of about 28 kDa. Cawston et al. (1981) Biochem. J. 195:159-165; and Murphy et al. (1991). TIMP-1 was the first inhibitor to be cloned from human tissue. Docherty et al. (1985) Nature 318:66-69; Gasson et al. (1985) Nature 315:768-771; and Carmichael et al. (1986) Proc. Natl. Acad. Sci. USA 83:2407-2411. TIMP-1 was subsequently cloned from murine, rabbit, bovine and porcine tissues. Edwards et al. (1986) Nucl. Acids Res. 14:8863-8878; Gewert et al. (1987) EMBO J. 6:651-657; Johnson et al. (1987) Mol. Cell. Biol. 77:2821-2829 (murine); Horowitz et al. (1989) J. Biol. Chem. 264:7092-7095 (rabbit); Freudenstein et al. (1990) Biochem. Biophys. Res. Commun. 171:250-256 (bovine); and Tanaka et al. (1992) Mol. Cell. Endocrinol. 83:65-71 (porcine).

TIMP-2 has been previously described. DeClerck et al. (1989) J. Biol. Chem. 264:17445-17453; Goldberg et al. (1989) Proc. Natl. Acad. Sci. USA 86:8207-8211; and Stetler-Stevenson et al. (1989) J. Biol. Chem. 264:17374-17378. TIMP-2 is a protein of approximately 21 kDa which is not N-glycosylated. TIMP-2 has been cloned and sequenced from human, bovine and murine sources. Boone et al. (1990) Proc. Natl. Acad. Sci. USA 87:2800-2804; Stetler-Stevenson et al. (1990) J. Biol. Chem. 265:13933-13938 (human); Boone et al. (1990) (bovine); and Leco et al. (1992) Gene 117:209-217; and Shimizu et al. (1992) Gene 114:291-292 (murine).

TIMP-1 and TIMP-2 from various species share identity in approximately 32% of residues. The first 22 N-terminal amino acids of the mature protein (i.e., following the site of signal peptide cleavage) is the most highly conserved region with 16 identical amino acid residues. This region appears to contain an active site,

with His^7 and Gln^9 likely being particularly important in the presumed interaction with the zinc at the active site. O'Shea et al. (1992) Biochem. 31:10146-10152. Also highly conserved are 12 cysteine residues.

In the case of TIMP-1, six disulfide bonds (Williamson et al. (1990) Biochem. J. 268:267-274) impose a secondary structure comprising two domains, each containing three loops (Murphy et al. (1991) Biochem. 30:8097-8102; and Woessner (1991)): a large three-loop, N-terminal domain possessing MMP-inhibiting activity and a small three-loop, C-terminal domain that is perhaps important in protein localization and/or complex formation with the progelatinases. Murphy et al. (1991). Both TIMP-1 and TIMP-2 bind to activated metalloproteinases in a 1:1 molar ratio and inhibit their enzyme activity. However, TIMP-1 and TIMP-2 form complexes with different inactive pro-enzymes: TIMP-1 with the pro-form of gelatinase B (MMP-9) (Wilhelm et al. (1989) J. Biol. Chem. 264:17213-17221; and Goldberg et al. (1992) J. Biol. Chem. 267:4583-4591) and TIMP-2 with the pro-form of gelatinase A (MMP-2). Goldberg et al. (1992) J. Biol. Chem. 267:4583-4591; and Stetler-Stevenson et al. (1990).

TIMPs are thought to play physiological roles in a variety of contexts: tissue remodeling and wound repair, including the prevention and repair of blood vessel injury and aneurysms, rheumatoid arthritis, various ulcerated conditions, e.g., resulting in the cornea as the result of alkali burns or as a result of infection by Pseudomonas aeruginosa, Acanthamoeba, Herpes simplex, and vaccinia viruses; periodontal disease; epidermolysis bullosa; promoting the growth and/or proliferation of, e.g., erythroid progenitors and a variety of cultured cells; tissue vascularization;

embryogenesis and blastocyst implantation; lactation; bone remodeling; oncogenic transformation; and cell migration, including the invasion and metastasis of cancer cells.

A third member of the TIMP family was recently identified in chicken embryo fibroblasts (CEF) and named ChIMP-3. Staskus et al. (1991) J. Biol. Chem. 266:449-454; and Pavloff et al. (1992) J. Biol. Chem. 267:17321-17326. ChIMP-3 was originally described as a protein whose synthesis is stimulated during the early stages of oncogenic transformation or after treatment of normal cells with phorbol myristate acetate. Blenis and Hawkes (1983) Proc. Natl. Acad. Sci. USA 80:770-774; and Blenis and Hawkes (1984) J. Biol. Chem. 259:11563-11570. This 21 kDa protein was purified from the extracellular matrix (ECM) of fibroblasts. Its NH_2-terminal sequence shows a strong similarity to the sequences of several mammalian TIMPs and it displays metalloproteinase inhibitor activity. Staskus et al. (1991). Based on these and other biochemical data, it was proposed that the protein was a TIMP variant or a third member of the TIMP family.

A human TIMP-3 heretofore unknown would be a useful addition to the TIMP family and help in modulating and fine-tuning metalloproteinase inhibition.

Means for diagnosing and treating conditions involving the activity of MMPs are highly desirable. The present invention addresses these and other needs.

Disclosure of the Invention

The present invention provides an isolated human TIMP-3 polypeptide having substantially the same amino acid residue sequence given in Figure 11.

The present invention also includes the nucleotide sequence of human TIMP-3 as depicted in Figure 11.

The present invention also provides antibodies specific for human TIMP-3, preferably monoclonal antibodies specific for human TIMP-3.

The present invention also includes a method of detecting the presence of human TIMP-3 in a sample, the method comprising the steps of: (a) contacting the sample with an antibody specific for human TIMP-3, under conditions suitable for the formation of a human TIMP-3-antibody complex; and (b) detecting the presence of the complex.

In another embodiment, the present invention provides a method of detecting elevated levels of human TIMP-3 in a test sample, the method comprising the steps of: (a) contacting the test sample with an antibody specific for human TIMP-3, under conditions suitable for the formation of a human TIMP-3-antibody complex; (b) contacting a control sample with the antibody, under conditions suitable for human TIMP-3-antibody complex formation; (c) detecting the level of human TIMP-3-antibody complex formation in the control sample and in the test sample; and (d) comparing the levels of complex formation in the control and test samples.

The present invention also provides a pharmaceutical composition comprising a human TIMP-3 polypeptide in a pharmaceutically acceptable excipient.

Brief Description of the Drawings

Figure 1 shows the primary structure of ChIMP-3. I, II, and III are potential sequences for the synthesis of peptides to be used for antibody production. A and B are targets for site-directed mutagenesis to

introduce glycosylation sites into ChIMP-3. Aspartate 16 (enclosed in box) is also a target for conversion to alanine.

Figure 2 shows protease/substrate SDS-PAGE analysis of human and chicken ECM. Lane 1 contains reduced and alkylated molecular weight standards: phosphorylase B (M_r=97,400), bovine serum albumin (M_r=66,200), ovalbumin (M_r=45,000), carbonic anhydrase (M_r=31,000), soybean trypsin inhibitor (M_r=21,500), and lysozyme (M_r=14,400). Lanes 2 and 3 are concentrated conditioned media (CM) from normal whole embryo cells (FHs 173We) equivalent to 300 μl and 150 μl, respectively. Lanes 4-11 contain ECM from human cell lines, as indicated. Lanes 4 and 5 are FHs 173We cells; lanes 6 and 7 are human embryonic kidney cells transformed by adenovirus type 5 DNA (293); lanes 8 and 9 are cells derived from a metastatic human neuroblastoma (SK-N-SH); lanes 10 and 11 are fibrosarcoma cells (Hs 913T); and lane 12 is ECM from approximately 4 x 10^4 chicken embryo fibroblasts (CEF) (lane 12).

Figure 3 shows protease/substrate SDS-PAGE analysis of conditioned media (left) and ECM (right) from the following cell lines: LA24-CEF (lane 1), SK-N-SH (lane 2), 293 (lane 3), mouse 3T3 (lane 5), and rat pheochromocytoma cells derived from an adrenal gland tumor (PC12) (lane 6). Molecular weight standards are in lane 4.

Figure 4 shows protease/substrate SDS-PAGE analysis of TIMPs present in conditioned media and ECM of mouse 3T3 cells and transforming chicken LA24-CEF cells. Lanes 1 and 3, CM; lanes 2 and 4, ECM; lane 5, molecular weight standards.

Figure 5 shows the nucleotide sequence of mouse TIMP-3 cDNA, aligned with that of ChIMP-3.

Figure 6 shows a comparison of the deduced amino acid sequence of mTIMP-3 with TIMP-1 from bovine, pig, human, rabbit, and mouse TIMP-1 (two sequences); with TIMP-2 from human, mouse and bovine; and with TIMP-3 from chicken. The mature mTIMP-3 protein begins with the "CTCSPS..." as marked.

Figure 7 shows a Western blot of human ECM and CM probed with anti-human TIMP-1, anti-ChIMP-3 and anti-human TIMP-2. Lanes 2, 3, and 5: ECM from human FHs 173We cells (approximately 2.4 x 10^6 cells); lanes 1 and 6: dialyzed and concentrated CM (equivalent to 60 μl CM) from FHs 173We cells; lane 4 is ECM from CEF cells (approximately 4 x 10^6 cells). The data in this figure were derived from three different gels and therefore the mobilities of the proteins cannot be compared directly. Using molecular weight standards on each individual gel (not shown), the apparent molecular weights of the reduced proteins are as follows: lane 1, TIMP-1 (30.4 kDa); lane 3, human TIMP-3 (24.7 kDa), minor band (28.5 kDa); lane 4, ChIMP-3 (24.5 kDa); lane 6, TIMP-2 (24.8 kDa).

Figure 8 is a Western blot of human TIMP-3 in a reduced and unreduced state probed with an antibody to the NH_2-terminus of ChIMP-3, showing the effect of β-mercaptoethanol on the electrophoretic migration of human TIMP-3 on SDS polyacrylamide gels. Lanes 1-4 contain ECM from approximately 2.4 x 10^6 FHs 173We cells; lane 6 contains ECM from approximately 4 x 10^6 CEF; lane 5 contains sample loading buffer only. (+) indicates addition of β-mercaptoethanol to a final concentration of 5% v/v; (-) indicates no addition of β-mercaptoethanol.

Figure 9 shows protease/substrate SDS-PAGE analysis of ECM from human SK-N-SH cells, either untreated (-) or treated with N-glycosidase-F (+).

Figure 10 shows a comparison of the N-terminal amino acid sequence of human TIMP-3 with chicken and mouse TIMP-3s and consensus sequences of TIMP-1 and TIMP-2.

Figure 11 shows cDNA nucleotide and amino acid sequences of human TIMP-3. The end of the signal peptide is denoted by the line and indication of mature TIMP-3.

Best Modes for Carrying Out the Invention

Described herein is the purification and cloning of human TIMP-3 and polypeptides, nucleic acids, antibodies, and diagnostic, therapeutic, prophylactic and other compositions and uses thereof. Human TIMP-3 was identified on the basis of its ECM localization, its molecular weight, lack of N-linked glycosylation, NH_2-terminal sequence, amino acid composition, and recognition by antibodies to pure ChIMP-3.

Degradation of the ECM by matrix metalloproteinases is highly controlled in normal physiological processes but appears to be accelerated in some pathological conditions. This is due in part to an imbalance between the proteinases and their naturally occurring inhibitors, the Tissue Inhibitors of MetalloProteinases (e.g., TIMP-1 and TIMP-2). TIMP-1 and TIMP-2 can be found free in the conditioned media of cultured cells.

Recently, a third member of this family, ChIMP-3 (chicken inhibitor of metalloproteinases-3), a 21.8 kDa protein, was isolated from chicken embryo fibroblasts. In contrast to TIMP-1 and TIMP-2, ChIMP-3 is located exclusively in the ECM. The ChIMP-3 cDNA sequence has recently been elucidated and indicates that, although the protein is structurally related to TIMP-1 and TIMP-2, with 42% sequence identity to a consensus sequence of

TIMP-2 and 28% to TIMP-1, it is clearly a distinct TIMP-3. Pavloff et al. (1992). The protein (M_r approximately 22 kDa, reduced) is intermediate in molecular weight between TIMP-1 and TIMP-2 and is not N-glycosylated.

Like TIMP-1 and TIMP-2, ChIMP-3 is a multifunctional protein which stimulates the division of serum-deprived cells. Yang and Hawkes (1992) Proc. Natl. Acad. Sci. USA 89:10676-10680. Furthermore, it promotes the expression of the transformed phenotype by enhancing detachment of transforming cells from the ECM and accelerating morphological transformation (Yang and Hawkes (1992)), properties which have not been described for TIMP-1 and TIMP-2. Additional distinct characteristics which set this protein apart from other members of the family include its relative insolubility and its specific localization in the ECM. Blenis and Hawkes (1983); Blenis and Hawkes (1984); and Staskus et al. (1991) J. Biol. Chem. 266:446-454. In contrast, TIMP-1 and TIMP-2 are soluble proteins found in body fluids and conditioned media of cultured cells and tissues. Murphy and Sellers (1980), In: Collagenases in Normal and Pathological Connective Tissues, eds. Woolley and Evanson, John Wiley & Sons Ltd., London, pp. 65-81; Cawston et al. (1981); Murphy et al. (1991); Reynolds et al. (1981) In: Cellular Interactions, eds. Dingle and Gordon, Elsevier/North-Holland, Amsterdam, pp. 205-213; Welgus and Stricklin (1983) J. Biol. Chem. 258:12259-12264; Stetler-Stevenson et al. (1989); Goldberg et al. (1989); Osthues et al. (1992) FEBS Lett. 296:16-20; and Moutsiakis et al. (1992) Connect. Tissue Res. 28:213-230).

The examples below describe the identification of mouse and human TIMP-3, the isolation of human TIMP-3, and the cloning and sequence of their genes. Human TIMP-

3 polypeptides, nucleic acids, and antibodies specific for human TIMP-3 polypeptides are useful for a host of uses, including the diagnosis, therapy, or prophylaxis of a variety of conditions characterized by excess or unwanted MMP activity.

Polypeptides

The term "TIMP-3" refers to a polypeptide or protein which (1) shows preferential association with the ECM; (2) displays TIMP activity as determined by protease/substrate SDS polyacrylamide gel electrophoresis; (3) is the only major band on protease/substrate SDS gel between 21 and 31 kDa; and (4) is recognized by antisera which specifically binds to TIMP-3, i.e., is capable of distinguishing TIMP-3 from other members of the TIMP family. As used herein, "human TIMP-3" is a polypeptide, or fragment thereof, substantially homologous to the mature TIMP-3 depicted in Figure 11. Human TIMP-3 may be isolated in its native form from human tissue or produced by recombinant DNA techniques. A polypeptide produced by the recombinant expression of a nucleic acid which encodes human TIMP-3 polypeptide is also considered a human TIMP-3, even if such a polypeptide is expressed in a non-human host cell. When produced recombinantly, the endogenous signal peptide may be used if expressed in eukaryotic expression systems. Human TIMP-3 may also be produced with any functional signal peptide, preferably another TIMP signal peptide in the case of eukaryotic expression systems. Recombinant human TIMP-3 may also be produced without a signal peptide.

As measured by protease/substrate gel analysis, human TIMP-3 is associated with the ECM (and not with conditioned media) and has an apparent molecular weight

of about 24 kDa. It is understood by those skilled in the art that this molecular weight is only approximate because it depends on a number of variables. The amino acid sequence of the mature polypeptide is 188 amino acids.

The bulk of human TIMP-3 appears to be non-N-glycosylated; however, there appears to be a glycosylation site near the C-terminus. Protease/substrate gel analysis also showed in the ECM of human cells minor TIMP species of 28-29 kDa in addition to the major 24 kDa species. These minor species appear to be N-glycosylated, and one or more may be N-glycosylated TIMP-3, which is also included in the invention. Human TIMP-3 appears to contain intramolecular disulfide bonds because it migrates differently in the presence of reducing agents. As used herein, human TIMP-3 encompasses non-glycosylated and glycosylated forms of the polypeptide. Preferably, the glycosylated forms are N-linked.

Encompassed by the claimed human TIMP-3 polypeptides are variants of human TIMP-3 in which there have been trivial substitutions, deletions, insertions or other modifications of the native human TIMP-3 polypeptide which substantially retain human TIMP-3 characteristics, particularly silent or conservative substitutions.

Conservative substitutions typically include substitutions within the following groups: glycine, alanine; valine, isoleucine, leucine; aspartic acid, glutamic acid; asparagine, glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

The polypeptides of the present invention may be coupled to a solid phase support, e.g., nitrocellulose, nylon, column packing materials (e.g.,

Sepharose beads), magnetic beads, glass wool, cells, or other substrates.

"Isolated" The terms "isolated," "pure," "substantially pure," and "substantially homogeneous" are used interchangeably to describe human TIMP-3 which has been separated from components which naturally accompany it. A monomeric protein is substantially pure when at least about 60 to 75% of a sample exhibits a single polypeptide sequence. A substantially pure protein will typically comprise about 60 to 90% W/W of a protein sample, more usually about 95%, and preferably will be over about 99% pure. Protein purity or homogeneity may be indicated by a number of means well known in the art, such as polyacrylamide gel electrophoresis of a protein sample, followed by visualizing a single polypeptide band upon staining the gel or chemically determining the N-terminal sequence by Edman degradation. For certain purposes, higher resolution may be provided by using reversed-phase high performance liquid chromatography (HPLC) or other means well known in the art for purification.

Human TIMP-3 is substantially free of naturally associated components when it is separated from the contaminants which accompany it in its natural state. Thus, a polypeptide which is chemically synthesized or expressed as a recombinant protein in a host cell different from the cell from which it naturally originates is substantially free of its naturally associated components.

A polypeptide produced as an expression product of an isolated and manipulated genetic sequence is an isolated polypeptide, as used herein, even if expressed in a homologous cell type. Synthetically made forms or

molecules expressed by heterologous cells are inherently isolated molecules.

Protein purification The present invention describes the purification of human TIMP-3 from human cells. Various methods for the isolation of human TIMP-3 from other biological material, such as from cells transformed with recombinant nucleic acids encoding human TIMP-3, may be accomplished by various methods well known in the art. For example, human TIMP-3 may be purified by immunoaffinity chromatography employing, e.g., the antibodies provided by the present invention. Various methods of protein purification are well known in the art, and include those described, e.g., in Guide to Protein Purification, ed. Deutscher, vol. 182 of Methods in Enzymology (Academic Press, Inc.: San Diego, 1990) and Scopes, Protein Purification: Principles and Practice (Springer-Verlag: New York, 1982).

Protein sequence determination To determine the actual amino acid sequence or to obtain polypeptide fragments of human TIMP-3, the protein may be digested with enzymes such as trypsin, clostripain, or Staphylococcus protease or with chemical agents such as cyanogen bromide, O-iodosobenzoate, hydroxylamine or 2-nitro-5-thiocyanobenzoate. Peptide fragments may be separated by HPLC and analyzed by gas-phase sequencing. Other sequencing methods known in the art may also be used.

Protein modifications; fragments The present invention also provides for human TIMP-3 polypeptides or fragments thereof which are substantially homologous to the primary structural sequence but which include, e.g., in vivo or in vitro chemical and biochemical modifications or which incorporate unusual amino acids. Such modifications include, for example, acetylation,

carboxylation, phosphorylation, glycosylation, ubiquitination, and labelling, e.g., with radionuclides and various enzymatic modifications. There are a variety of methods for labelling polypeptides and of substituents or labels useful for such purposes, including radioactive isotopes such as ^{32}P, ligands which bind to labeled antiligands (e.g., antibodies), fluorophores, chemiluminescent agents, enzymes, and antiligands which can serve as specific binding pair members for a labeled ligand. The choice of label depends on the sensitivity required, ease of conjugation with the primer, stability requirements, and available instrumentation. Methods of labelling polypeptides are well known in the art. See, e.g., Molecular Cloning: A Laboratory Manual, 2nd ed., Vol. 1-3, ed. Sambrook, et al., Cold Spring Harbor Laboratory Press (1989); or Current Protocols in Molecular Biology, ed. Ausubel et al., Greene Publishing and Wiley-Interscience: New York, 1987 and periodic updates.

Besides substantially full-length polypeptides, the present invention provides fragments of human TIMP-3 which retain at least one of the biological activities characteristic of human TIMP-3, such as antigenic fragments useful for raising human TIMP-3-specific antibodies. A polypeptide "fragment," "portion," or "segment" is a stretch of amino acid residues of at least about 7-17 amino acids (or the minimum size retaining an antigenic determinant of human TIMP-3 and capable of raising human TIMP-3-specific antibodies) and preferably substantially full length. Significant biological activities include immunological activity, MMP inhibition, and other biological activities characteristic of human TIMP-3. As used herein, both supra and infra, "human TIMP-3" encompasses such

fragments. Immunological activities include both immunogenic function in a target immune system, as well as sharing of immunological epitopes for binding, serving as either a competitor or substitute antigen for a human TIMP-3 epitope.

For immunological purposes, tandemly repeated human TIMP-3 fragments may be used as immunogens, thereby producing highly antigenic proteins. Alternatively, such polypeptides will serve as highly efficient competitors for specific binding. Production of antibodies specific for human TIMP-3 is described below.

The present invention also provides for fusion polypeptides comprising human TIMP-3. Homologous polypeptides may be fusions between two or more human TIMP-3 sequences or between the sequences of human TIMP-3 and a related protein. Likewise, heterologous fusions may be constructed which would exhibit a combination of properties or activities of the derivative proteins. For example, ligand-binding or other domains may be "swapped" between different new fusion polypeptides or fragments. Such homologous or heterologous fusion polypeptides may display, for example, altered strength or specificity of binding. Fusion partners include immunoglobulins, bacterial β-galactosidase, trpE, protein A, β-lactamase, alpha amylase, alcohol dehydrogenase and yeast alpha mating factor. See, e.g., Godowski et al. (1988) Science 241:812-816.

Fusion proteins are typically made by either recombinant nucleic acid methods, as described below, but may be chemically synthesized. Techniques for synthesis of polypeptides are described, for example, in Merrifield (1963) J. Amer. Chem. Soc. 85:2149-2156.

Figure 1 shows the primary structure of ChIMP-3 based on a model for TIMP-1 by Woessner (1991). The

numbering of the loops is according to Murphy et al. (1991). I, II, and III in Figure 1 are potential sequences for the synthesis of peptides to be used for antibody production. A and B are targets for site-directed mutagenesis to introduce glycosylation sites into ChIMP-3. Aspartate 16 (enclosed in box) is also a target for conversion to alanine. Similarly, knowledge of the structure of human TIMP-3 based on the present disclosure will enable those skilled in the art to design peptides for antibody production, to introduce glycosylation sites or to perform site-directed mutagenesis, as desired.

Nucleic Acids

The term "human TIMP-3" when applied to a nucleic acid refers to a nucleic acid which encodes a human TIMP-3 polypeptide. The nucleic acids of the present invention will possess a sequence which is either derived or substantially similar to the native human TIMP-3-encoding gene as shown in Figure 11.

The DNA compositions of this invention include RNA, cDNA, genomic DNA, synthetic forms, and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified or contain non-natural or derivatized nucleotide bases. Recombinant nucleic acids comprising sequences otherwise not naturally occurring are also provided by this invention. Although the wild type sequence may be employed, the wild type sequence will often be altered, e.g., by deletion, substitution, or insertion. cDNA or genomic libraries of various types may be screened as natural sources of the nucleic acids of the present invention, or such nucleic acids may be provided by amplification of sequences resident in genomic DNA or other natural sources, e.g.,

by the polymerase chain reaction (PCR). See, e.g., PCR Protocols: A Guide to Methods and Applications, Innis, M., et al., eds., Academic Press: San Diego (1990). The choice of cDNA libraries normally corresponds to a tissue source which is abundant in mRNA for the desired receptors. Phage libraries are normally preferred, but plasmid libraries may also be used. Clones of a library are spread onto plates, transferred to a substrate for screening, denatured and probed for the presence of desired sequences.

The DNA sequences used in this invention will usually comprise at least about 5 codons (15 nucleotides), more usually at least about 7 to 15 codons, and most preferably at least about 35 codons. One or more introns may also be present. This number of nucleotides is usually about the minimal length required to hybridize specifically with a human TIMP-3-encoding sequence.

Techniques for nucleic acid manipulation are described generally, for example, in Sambrook et al. (1989) or Ausubel et al. (1987). Reagents useful in applying such techniques, such as restriction enzymes and the like, are widely known in the art and commercially available from such vendors as New England BioLabs, Boehringer Mannheim, Amersham, Promega Corp., U. S. Biochemicals, New England Nuclear, and a number of other sources. The recombinant nucleic acid sequences used to produce the proteins of the present invention may be derived from natural or synthetic sequences. Many natural gene sequences are obtainable from various cDNA or from genomic libraries using appropriate probes. See, GenBank, National Institutes of Health.

"Isolated" or "pure" An "isolated" or "purified" nucleic acid is a nucleic acid, e.g., an RNA,

DNA, or a mixed polymer, which is substantially separated from other DNA sequences which naturally accompany a native human sequence, e.g., ribosomes, polymerases, and many other human genome sequences. The term embraces a nucleic acid sequence which has been removed from its naturally occurring environment, and includes recombinant or cloned DNA isolates and chemically synthesized analogues or analogues biologically synthesized by heterologous systems.

"Encode" A nucleic acid is said to "encode" a polypeptide if, in its native state or when manipulated by methods well known to those skilled in the art, it can be transcribed and/or translated to produce the polypeptide or a fragment thereof. The anti-sense strand of such a nucleic acid is also said to encode the sequence.

"Operably linked" A nucleic acid sequence is operably linked when it is placed into a functional relationship with another nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter affects its transcription or expression. Generally, operably linked means that the DNA sequences being linked are contiguous and, where necessary to join two protein coding regions, contiguous and in reading frame.

"Recombinant" The term "recombinant" nucleic acid is one which is not naturally occurring, or is made by the artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by either chemical synthesis means, or by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques. Such is usually done to replace a codon with a redundant codon encoding the same or a conservative amino acid,

while typically introducing or removing a sequence recognition site. Alternatively, it is performed to join together nucleic acid segments of desired functions to generate a desired combination of functions.

Probes and primers Nucleic acid probes and primers may be prepared based on the sequence of the human TIMP-3 cDNA sequences provided by the present invention. The probe or primer may comprise an isolated nucleic acid, and, especially in the case of probes, are typically attached to a label or reporter molecule. Probes may be used to identify the presence of a hybridizing nucleic acid sequence, e.g., a human TIMP-3 mRNA in a tissue or other sample or a human TIMP-3 cDNA or genomic clone in a library. Primers may be used, for example, for amplification of nucleic acid sequences, e.g., by the polymerase chain reaction (PCR). The preparation and use of probes and primers is described, e.g., in Sambrook et al. (1989) or Ausubel et al. (1987).

Antisense nucleic acids capable of specifically binding to human TIMP-3 sequences are also useful for interfering with gene expression and therefore may be useful for therapeutic or prophylactic compositions. See, e.g., EPO publication 431523A2.

Nucleic acids encoding human TIMP-3 include not only a native or wild-type human TIMP-3 sequence but also any sequence capable of encoding a human TIMP-3 polypeptide, which may be synthesized by making use of the redundancy in the genetic code. Various codon substitutions may be introduced, e.g., silent or conservative changes thereby producing various endonuclease restriction sites or to optimize expression for a particular system.

The nucleic acids of the present invention may be derived from naturally occurring or recombinant single

or double stranded nucleic acids or may be chemically synthesized.

Portions of the DNA sequence having at least about 15 contiguous nucleotides from a DNA sequence encoding a human TIMP-3 are preferred as probes or primers.

Preparation of recombinant or chemically synthesized nucleic acids; vectors, transformation, host cells Large amounts of the nucleic acids of the present invention may be produced by replication in a suitable host cell. The natural or synthetic DNA fragments coding for a desired fragment will be incorporated into recombinant nucleic acid constructs, typically DNA constructs, capable of introduction into and replication in a prokaryotic or eukaryotic cell. Usually the DNA constructs will be suitable for replication in a unicellular host, such as yeast or bacteria, but may also be intended for introduction into, with and without and integration within the genome, cultured mammalian or plant or other eukaryotic cell lines. The purification of nucleic acids produced by the methods of the present invention are described, e.g., in Sambrook et al. (1989) or Ausubel et al. (1987).

The nucleic acids of the present invention may also be produced by chemical synthesis, e.g., by the phosphoramidite method described by Beaucage and Carruthers (1981) Tetra. Letts. 22:1859-1862 or the triester method according to Matteucci et al. (1981) J. Am. Chem. Soc. 103:3185-3191, and may be performed on commercial automated oligonucleotide synthesizers. A double-stranded fragment may be obtained from the single stranded product of chemical synthesis either by synthesizing the complementary strand and annealing the strands together under appropriate conditions or by

adding the complementary strand using DNA polymerase with an appropriate primer sequence.

DNA constructs prepared for introduction into a prokaryotic or eukaryotic host typically comprise a replication system recognized by the host, including the intended DNA fragment encoding the desired polypeptide, and will preferably also include transcription and translational initiation regulatory sequences operably linked to the polypeptide encoding segment. Expression vectors include, for example, an origin of replication or autonomously replicating sequence (ARS) and expression control sequences, a promoter, an enhancer and necessary processing information sites, such as ribosome-binding sites, RNA splice sites, polyadenylation sites, transcriptional terminator sequences, and mRNA stabilizing sequences. Secretion signals may also be included where appropriate, whether from a native human TIMP-3 protein or from other receptors or from secreted polypeptides of the same or related species, which allow the protein to cross and/or lodge in cell membranes, and thus attain its functional topology, or be secreted from the cell. Such vectors are prepared by means of standard recombinant techniques well known in the art and discussed, for example, in Sambrook et al. (1989) or Ausubel et al. (1987).

An appropriate promoter and other necessary vector sequences is selected so as to be functional in the host, and may, when appropriate, include those naturally associated with human TIMP-3 genes. Examples of workable combinations of cell lines and expression vectors are described in Sambrook et al. (1989) or Ausubel et al. (1987); see also, e.g., Metzger et al. 1988) Nature 334:31-36. Many useful vectors are known in the art and may be obtained from such vendors as

Stratagene, New England Biolabs, Promega Corp., and others. Promoters such as the trp, lac and phage promoters, tRNA promoters and glycolytic enzyme promoters may be used in prokaryotic hosts. Useful yeast promoters include the promoter regions for metallothionein, 3-phosphoglycerate kinase or other glycolytic enzymes such as enolase or glyceraldehyde-3-phosphate dehydrogenase, enzymes responsible for maltose and galactose utilization, and others. Suitable vectors and promoters for use in yeast expression are further described in Hitzeman et al. EP 73,657A. Appropriate non-native mammalian promoters might include the early and late promoters from SV40 (Fiers et al. (1978) Nature 273:113) or promoters derived from murine Molony leukemia virus, mouse mammary tumor virus, avian sarcoma viruses, adenovirus II, bovine papilloma virus or polyoma. In addition, the construct can be joined to an amplifiable gene (e.g., DHFR) so that multiple copies of the gene are made. For appropriate enhancer and other expression control sequences see also Enhancers and Eukaryotic Gene Expression, Cold Spring Harbor Press: N.Y., 1983.

While such expression vectors preferably replicate autonomously, they may also be inserted into the genome of the host cell.

Expression and cloning vectors generally contain a selectable marker, a gene encoding a protein necessary for the survival or growth of a host cell transformed with the vector. The presence of this gene ensures the growth of only those host cells which express the inserts. Typical selection genes encode proteins that (a) confer resistance to antibiotics or other toxic substances, e.g. ampicillin, neomycin, methotrexate, etc.; (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex

media, e.g. the gene encoding D-alanine racemase for Bacilli. The choice of the proper selectable marker depends on the host cell, and appropriate markers for different hosts are well known in the art.

The vectors containing the nucleic acids of interest can be transcribed in vitro and the resulting RNA introduced into the host cell by well known methods (e.g., by injection; see, Kubo et al. (1988) FEBS Lett. 241:119-125), or the vectors can be introduced directly into host cells by methods well known in the art, which vary depending on the type of cellular host, including electroporation; transfection employing calcium chloride, rubidium chloride, calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; infection (where the vector is an infectious agent, such as a retroviral genome); and other methods. See generally, Sambrook et al. (1989) and Ausubel et al. (1987). The cells into which have been introduced nucleic acids described above are meant to also include the progeny of such cells.

Large quantities of the nucleic acids and polypeptides of the present invention can be prepared by expressing the human TIMP-3 nucleic acids or portions thereof in vectors or other expression vehicles in compatible prokaryotic or eukaryotic host cells. The most commonly used prokaryotic hosts are strains of Escherichia coli, although other prokaryotes, such as Bacillus subtilis or Pseudomonas may also be used.

Mammalian or other eukaryotic host cells, such as those of yeast, filamentous fungi, plant, insect, amphibian or avian species, are also useful for production of the proteins of the present invention. See, Tissue Culture, Kruse and Patterson, ed., Academic Press (1973). Examples of commonly used mammalian host

cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cells, and WI38, BHK, and COS cell lines, or others which may be appropriate, e.g., to provide higher expression, desirable glycosylation patterns, or other features.

Transformed cells are selected by using markers depending on the mode of the vector construction. The marker may be on the same or a different DNA molecule, preferably the same DNA molecule. In prokaryotic hosts the transformant can be selected, e.g., by resistance to ampicillin, tetracycline or other antibiotics. Production of a particular product based on temperature sensitivity can also serve as an appropriate marker.

Prokaryotic or eukaryotic cells transformed with the nucleic acids of the present invention are useful not only for the production of the nucleic acids and polypeptides of the present invention, but also, for example, in studying the characteristics of human TIMP-3 polypeptides.

Two constructs of ChIMP-3 cDNA have been expressed as fusion proteins in Escherichia coli. In Western blot analysis of induced bacterial proteins in four strains, polyclonal anti-ChIMP-3 specifically detects proteins at approximately 32-35 kDa, the expected molecular weight for ChIMP-3 plus the fusion partner. These results indicate that human TIMP-3 polypeptides may also be expressed in E. coli.

Antibodies

The present invention also provides polyclonal and/or monoclonal antibodies capable of specifically binding to human TIMP-3 produced by in vitro or in vivo techniques well known in the art. Such antibodies are raised against human TIMP-3 and are capable of

distinguishing human TIMP-3 from other polypeptides, including human TIMP-1 or TIMP-2. Preferably, such antibodies also are capable of distinguishing human TIMP-3 from TIMP-3 of other species under conditions suitable for complex formation.

Peptide fragments suitable for raising antibodies may be prepared by chemical synthesis, and are commonly coupled to a carrier molecule (e.g., keyhole limpet hemocyanin) and injected into a mammal, e.g., rabbits, over several months. The rabbit sera is tested for immunoreactivity to human TIMP-3. Monoclonal antibodies are made by injecting the animal, e.g., mice with the protein, polypeptides, fusion proteins or fragments thereof. Monoclonal antibodies are screened by ELISA and tested for specific immunoreactivity with human TIMP-3. See, Harlow and Lane (1988) Antibodies: A Laboratory Manual, CSH Laboratories. These antibodies are useful in diagnostics such as immunoassays as well as in pharmaceuticals.

For production of polyclonal antibodies, an appropriate target immune system is selected, typically a mouse or rabbit. The substantially purified antigen is presented to the immune system in a fashion determined by methods appropriate for the animal and other parameters well known to immunologists. Typical sites for injection are in the footpads, intramuscularly, intraperitoneally, or intradermally.

An immunological response is usually assayed with an immunoassay. Normally such immunoassays involve some purification of a source of antigen, for example, produced by the same cells and in the same fashion as the antigen was produced. A variety of immunoassay methods are well known in the art. See, e.g., Harlow and Lane

(1988); and Goding (1986) Monoclonal Antibodies: Principles and Practice, 2d ed, Academic Press, New York.

Monoclonal antibodies with affinities of 10^8 M^{-1} preferably 10^9 to 10^{10}, or stronger are typically made by standard procedures as described, e.g., in Harlow and Lane (1988) or Goding (1986). Briefly, appropriate animals are selected and the desired immunization protocol followed. After the appropriate period of time, the spleens of such animals are excised and individual spleen cells fused, typically, to immortalized myeloma cells under appropriate selection conditions. Thereafter the cells are clonally separated and the supernatants of each clone are tested for their production of an appropriate antibody specific for the desired region of the antigen.

Other suitable techniques involve in vitro exposure of lymphocytes to the antigenic polypeptides or alternatively to selection of libraries of antibodies in phage or similar vectors. See Huse et al. (1989) Science 246:1275-1281.

Frequently, the polypeptides and antibodies are labeled by joining, either covalently or noncovalently, a substance which provides for a detectable signal. A wide variety of labels and conjugation techniques are well known. Suitable labels include radionuclides, enzymes, substrates, cofactors, inhibitors, fluorescent agents, chemiluminescent agents, magnetic particles and the like. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241. Also, recombinant immunoglobulins may be produced, see Cabilly, U.S. Patent No. 4,816,567.

Methods of Use: Drug Screening

The present invention encompasses the use of human TIMP-3 in a variety of drug screening techniques. For drug screens, human TIMP-3 is provided free in solution, affixed to a solid support, or borne on a cell surface. One method of drug screening utilizes eukaryotic or prokaryotic host cells which are stably transformed with recombinant nucleic acids expressing human TIMP-3, preferably in competitive binding assays. Such cells, either in viable or fixed form, can be used for standard binding assays. One measures, for example, the formation of complexes between human TIMP-3 and the agent being tested, or examines the degree to which the formation of a complex between human TIMP-3 and a MMP is interfered with by the agent being tested.

Thus, the present invention provides methods of screening candidate drugs comprising contacting a sample containing the candidate drug with human TIMP-3 and assaying (i) for the presence of a complex between the drug and human TIMP-3, or (ii) for the presence of a complex between human TIMP-3 and a MMP. In such competitive binding assays human TIMP-3 is typically labeled. Free human TIMP-3 is separated from that present in a protein:protein complex, and the amount of free (i.e., uncomplexed) label is a measure of the binding of the agent being tested to human TIMP-3 or its interference with TIMP-3:MMP binding, respectively.

Another technique for drug screening provides high throughput screening for compounds having suitable binding affinity to human TIMP-3 and is described, e.g., in Geysen, European Patent Application 84/03564. Briefly stated, large numbers of different small peptide test compounds are synthesized on a solid substrate. The

peptide test compounds are reacted with labeled human TIMP-3 and washed. Bound human TIMP-3 is then detected.

The present invention also contemplates the use of competitive drug screening assays in which antibodies capable of specifically binding to human TIMP-3 compete with a test compound for binding to human TIMP-3. In this manner, the antibodies are used to detect the presence of any peptide which shares one or more antigenic determinants of human TIMP-3.

Methods of Use: Rational Drug Design

The goal of rational drug design is to produce structural analogues of biologically active polypeptides of interest or of small molecules with which they interact, e.g., agonists, antagonists, inhibitors, in order to fashion drugs which are, e.g., more active or stable forms of the polypeptide, or which, e.g., enhance or interfere with the function of a polypeptide in vivo. See, e.g., Hodgson (1991) Bio/Technology 9:19-21.

In one approach, one first determines the three-dimensional structure of a protein of interest or, for example, of a protein-inhibitor complex, by X-ray crystallography, by computer modelling or, most typically, by a combination of the two approaches. Less often, useful information regarding the structure of a polypeptide may be gained by modelling based on the structure of homologous proteins. An example of rational drug design is the development of HIV protease inhibitors. Erickson et al. (1990) Science 249:527-533.

It is also possible to isolate a target-specific antibody, select a functional assay and then solve its crystal structure. This approach, in principle, yields a pharmacore upon which subsequent drug design can be based. It is possible to bypass protein

crystallography altogether by generating anti-idiotypic antibodies (anti-ids) to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of the anti-ids would be expected to be an analogue of the original receptor. The anti-id could then be used to identify and isolate peptides from banks of chemically or biologically produced banks of peptides. The selected peptides would then act as the pharmacore.

Thus, one may design drugs which have, e.g., improved TIMP-3 activity or stability or which act as inhibitors, agonists, antagonists, etc. of TIMP-3. By virtue of the present invention, sufficient amount of polypeptide may be made available to perform such analytical studies as X-ray crystallography. In addition, the knowledge of the human TIMP-3 protein sequences provided herein will guide those employing computer modelling techniques in place of or in addition to X-ray crystallography.

Methods of Diagnosis

As set forth above, a number of human conditions are known to be mediated by excess or undesired matrix metalloproteinase activity. These include tumor metastasis, rheumatoid arthritis, ulcerations, particularly of the cornea, reaction to infection, periodontal disease, osteoporosis, and the like. It has been shown that TIMP-3 activity is induced, for example, by inflammatory cytokines and growth factors at a site of injury. TIMP-3 activity is also increased at an early stage of oncogenic transformation.

The present invention thus encompasses the diagnosis of the above named conditions and others characterized by excess or undesired MMP activity. Antibodies specific for human TIMP-3, preferably

monoclonal antibodies, may be used to determine levels of human TIMP-3 in fixed tissue sections by immunohistochemical methods or in a body fluid or tissue sample by ELISA or radioimmunoassays, for example. Oligonucleotide probes or primers based on the human TIMP-3 sequences may be useful for assaying elevated transcription of TIMP-3, e.g., by probing Northern blots, in situ hybridization or quantitative nucleic acid amplification methods such as the polymerase chain reaction (PCR), for example.

The claimed anti-human TIMP-3 antibodies are useful in monitoring therapy or prophylaxis regimens involving the pharmaceutical compositions of the present invention. Suitable samples, such as those derived from biopsied tissues, blood, serum, urine, or saliva, can be tested for the presence of the administered inhibitor at various times during the treatment protocol using standard immunoassay techniques which employ the claimed antibody preparations.

The ability of human TIMP-3 to bind MMPs may be exploited to determine the locations of excess amounts of one or more MMPs in situ. Similarly, the claimed antibodies specific for human TIMP-3 may be used to map the location of TIMP-3. When coupled to labels such as scintigraphic labels, e.g., technetium 99 or I-131, by standard coupling methods, human TIMP-3 may be administered to subjects for this purpose. These techniques can also be employed in histological procedures and in competitive immunoassays.

Methods of Use: Therapy and Prophylaxis

Human TIMP-3 polypeptides and nucleic acids are likewise useful for the treatment or prophylaxis of these conditions. These include, but are not limited to,

neoplasias such as fibrosarcomas, terato
neoplasias of epithelial origin, tumor me
inflammatory disorders such as rheumatoi
ulcerations, particularly of the cornea,
infection, periodontal disease, osteopor
like. Thus, the present invention encompasses pharmaceutical compositions comprising human TIMP-3 in one or more non-toxic pharmaceutically acceptable carriers and/or diluents and/or adjuvants. Other active ingredients may also be included in the compositions of the invention. Standard pharmaceutical formulation techniques are used, such as those disclosed in Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, PA, latest edition.

Such pharmaceutical formulations may be applied to a wound or ulcer or injected into a cancer or an arthritic joint, thereby inhibiting MMP activity. Anti-sense nucleic acids comprising sequences derived from a human TIMP-3 gene or antibodies specific for that gene are also useful in inhibiting human TIMP-3 expression.

The administration of human TIMP-3 is beneficial in tissue and wound repair (for example, in ulcers of the eye); promoting growth and/or proliferation of erythroid progenitors and a variety of cultured cells; tissue vascularization; and embryogenesis, blastocyst formation and other stages of pregnancy as well as lactation.

The potency of the claimed pharmaceutical compositions as inhibitors of MMP activity may be determined as described in U.S. Patent No. 5,183,900, U.S. Patent No. 4,743,587, and WO 92/13831, which reference Cawston et al. (1979) Anal. Biochem. 99:340-345; and Weingarten et al. (1986) Biochem. Biophys. Res. Comm. 139:1184-1187; or other tests of MMP inhibition

known in the art. Isolated MMP enzymes can be used to confirm the inhibiting activity of the claimed human TIMP-3 compositions, or crude extracts which contain the range of enzymes exhibiting MMP activity. Human TIMP-3 activity may also be assayed using the cell detachment and cell proliferation assays provided in the Examples below. For example, see Yang and Hawkes (1992).

The compositions of the present invention may be formulated for administration by any route, depending on the disease being treated. The compositions may be in the form of tablets, capsules, powders, granules, lozenges, liquid or gel preparations, such as oral, topical, or sterile parenteral solutions or suspensions.

Tablets and capsules for oral administration may be in a form suitable for unit dose presentation and may contain conventional excipients. Examples of these are binding agents such as syrup, acacia, gelatin, sorbitol, tragacanth, and polyvinylpyrrolidone; fillers such as lactose, sugar, maize-starch, calcium phosphate, sorbitol or glycine; tableting lubricants, such as magnesium stearate, talc, polyethylene glycol or silica; disintegrants, such as potato starch; or acceptable wetting agents, such as sodium lauryl sulfate. The tablets may be coated according to methods well known in normal pharmaceutical practice. Oral liquid preparations may be in the form of, for example, aqueous or oily suspensions, solutions, emulsions, syrups or elixirs, or may be presented as a dry product for reconstitution with water or other suitable vehicle before use. Such liquid preparations may contain conventional additives such as suspending agents, e.g., sorbitol, syrup, methyl cellulose, glucose syrup, gelatin, hydrogenated edible fats, emulsifying agents, e.g., lecithin, sorbitan monooleate, or acacia; non-aqueous vehicles (including

edible oils), e.g., almond oil, fractionated coconut oil, oily esters such as glycerine, propylene glycol, or ethyl alcohol; preservatives such as methyl or propyl p-hydroxybenzoate or sorbic acid, and, if desired, conventional flavoring or coloring agents.

For topical application to the skin, the drug may be made up into a cream, lotion, or ointment using conventional formulations. For topical applications to the eye, the drug may be made up into a solution or suspension in a suitable sterile aqueous or non-aqueous vehicle. Additives may also be included, e.g., buffers such as sodium metabisulphite or disodium edetate; preservatives such as bactericidal and fungicidal agents, including phenyl mercuric acetate or nitrate, benzalkonium chloride or chlorhexidine, and thickening agents, such as hypromellose.

The claimed pharmaceutical compositions may also be administered parenterally in a sterile medium. The drug may be dissolved or suspended in the vehicle, depending of the vehicle or concentration used. Adjuvants such as local anesthetics, preservatives, and buffering agents can also be dissolved in the vehicle. Commonly used excipients for injectable forms of the pharmaceutical compositions of the present invention include physiological saline, Hank's solution, Ringer's solution, and the like. Injection can be, e.g., intravenous, intramuscular, intraperitoneal, or subcutaneous.

For treatment of rheumatoid arthritis, for example, the claimed pharmaceutical compositions may be administered orally or injected into the affected joint.

The claimed pharmaceutical compositions may also be administered by transdermal or transmucosal delivery by including agents which effect penetration of

these tissues, such as bile salts, fusidic acid derivatives, cholic acid, and the like.

The use of targeting ligands allows the claimed pharmaceutical compositions to be delivered to specific locations. For example, to focus human TIMP-3 to inhibit the activity of matrix metalloproteinases in a tumor, the TIMP-3 may be conjugated to an antibody or fragment thereof which is immunoreactive with a tumor marker. The targeting ligand can also be a ligand which is specifically bound by a receptor which is present at the target site. Methods for coupling proteins such as human TIMP-3 are well known.

The dosage unit required can be determined by one of skill in the art and depends, for example, on the condition treated, nature of the formulation, nature of the condition, embodiment of the claimed pharmaceutical compositions, mode of administration, and condition and weight of the patient.

The invention will be better understood by reference to the following examples, which are intended to merely illustrate the best mode now known for practicing the invention. The scope of the invention is not to be considered limited thereto, however.

EXAMPLES

Example 1 -- Polyclonal Antibodies Against ChIMP-3

Polyclonal anti-ChIMP-3 antibodies were raised against ChIMP-3 from transforming chicken embryo fibroblasts purified to homogeneity by the methods described in Staskus et al. (1991); and Yang and Hawkes (1992). The polyclonal antibodies to ChIMP-3 were raised in a single white New Zealand rabbit following two

injections of ChIMP-3 (20 μg protein/immunization). A titer of 1:3000, as determined by ELISA using pure ChIMP-3, remained constant for three subsequent bleeds.

The antibodies recognized denatured and denatured/reduced ChIMP-3 on Western blots, performed as described below. The anti-ChIMP-3 antiserum detected a single, strong band at about 24 kDa on blots of chicken reduced ECM proteins from transforming chicken embryo fibroblasts (CEF). Occasionally, weakly reacting bands at approximately 26 kDa, 42 kDa, and 90-95 kDa were detected. Additional evidence indicates the human TIMP-3 has a propensity for aggregation, so the presence of multimers is not unexpected. Electroelution of the 42 kDa band from a preparative gel and subsequent analysis on a protease/substrate gel detects inhibitor activity in a band of protein at an M_r of about 24,000. The 90-95 kDa band could represent a complex between ChIMP-3 and the gelatinase that it inhibits. This assumes that such a complex would be resistant to dissociation in SDS. Indeed, such complexes are maintained in the presence of SDS if the samples are not heated prior to electrophoresis. De Clerck et al. (1991) J. Biol. Chem. 266:3893-3899.

Polyclonal ChIMP-3 antisera also recognized mouse TIMP-3 on Western blots of samples of ECM from mouse C3H 10T1/2 cells. The antibody reacts positively with a band at approximately 23-24 kDa (the only band recognized between 21 and 31 kDa) as well as a minor but recognizable band at approximately 55 kDa.

When used to probe Western blots, ChIMP-3 polyclonal antisera also recognized human TIMP-3 and an unidentified protein with a molecular weight of approximately 28-29 kDa.

Example 2 -- Polyclonal Antisera Against an N-terminal ChIMP-3 Peptide

The difficulty of generating sufficient amounts of pure ChIMP-3 or human TIMP-3 for structural, functional and immunological studies encouraged the development of an alternative strategy for the production of antigen. A peptide corresponding to N-terminal residues 3-13 of ChIMP-3 was synthesized: NH_2-Cys-Val-Pro-Ile-His-Pro-Gln-Asp-Ala-Phe-Cys-COOH. The peptide was synthesized by solid phase synthesis (yield: 120 mg) and sequenced by the Edman technique. It was coupled to both keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) and used to immunize white New Zealand rabbits (under contract with the Berkeley Antibody Company). Sera were analyzed by ELISA using the alternate hapten carrier conjugate, in each case, as the screening antigen. The KLH-peptide was the most immunogenic and provided antisera with titers in the range of 10,000-12,000. These antibodies recognize both ChIMP-3 and ChIMP-a on Western blots of ECM proteins from transforming CEF. As the NH_2-termini of this family of proteins are so similar, this result is not surprising. Whether these anti-peptide antibodies recognize either "native" ChIMP-3 or ChIMP-a has not yet been tested. This antibody also recognized human TIMP-3 and an unidentified protein with a molecular weight of approximately 28-29 kDa.

Example 3: Protease/Substrate Gel Electrophoresis of Human and Mouse TIMP-3

Metalloproteinase inhibitory activities in ECM and conditioned media (CM) from a large number of human or mouse cell lines were screened by protease/substrate SDS-PAGE, essentially as described in Staskus et al. (1991). Molecular weight standards for electrophoresis

included phosphorylase B (M_r=97,400), BSA (M_r=66,200), ovalbumin (M_r=45,000), carbonic anhydrase (M_r=31,000), soybean trypsin inhibitor (M_r=21,500), and lysozyme (M_r=14,400) (Bio-Rad, Richmond, CA). The protein set was denatured in 6 M guanidine HCl, reduced with β-mercaptoethanol, and acetylated with iodoacetamide to block sulfhydryl groups. The standards were then dialyzed against 62.5 mM Tris-HCl (pH 6.8) and the protein aggregate was dissolved by addition of SDS to a concentration of 10 mg/ml. The reduced and alkylated standards migrated identically to the untreated standards under reducing conditions of electrophoresis and provided discrete bands under the nonreducing conditions required for protease/substrate gels.

Figures 2, 3, and 4 show the results of the testing of several human and mouse cell lines by protease/substrate SDS-PAGE, demonstrating the existence of both human and mouse TIMP-3. Using protease/substrate gel electrophoresis, TIMP-3 activity has also been observed in mouse 3T3 and mouse C3H 10T1/2 cells.

A variety of cultured human cells have been screened by protease/substrate SDS-PAGE analysis (modified reverse zymography) in search of the human counterpart for ChIMP-3. A unique metalloproteinase inhibitor has been detected that is localized specifically in the ECM of the following normal, transformed and cancer cell lines: normal whole embryo (FHs 173We), normal gingival fibroblasts (GF11 and 1292), transformed primary embryonic kidney (293), neuroblastoma (SK-N-SH), carcinoma (HeLa S3), colon adenocarcinoma (Caco-2), ileocecal adenocarcinoma (HCT-8) and fibrosarcomas (SW 684 and Hs 913T).

Figure 2 shows protease/substrate SDS-PAGE analysis of ECM from a representative sample of human

cell lines and from chicken. Lane 1 contains reduced and alkylated molecular weight standards: phosphorylase B (M_r=97,400), BSA (M_r=66,200), ovalbumin (M_r=45,000), carbonic anhydrase (M_r=31,000), soybean trypsin inhibitor (M_r=21,500), and lysozyme (M_r=14,400). Lanes 2 and 3 are concentrated conditioned media (CM) from FHs 173We cells equivalent to 300 μl and 150 μl, respectively. Lanes 4-11 contain ECM from human cell lines, as indicated. Lanes 4 and 5 are FHs 173We cells; lanes 6 and 7 are 293 cells; lanes 8 and 9 are SK-N-SH cells; lanes 10 and 11 are Hs 913T cells. In each set of two lanes the sample on the left was produced by approximately 8 x 10^3 cells and the one on the right by 4 x 10^3 cells. Lane 12 is ECM from approximately 4 x 10^4 chicken embryo fibroblasts (CEF).

Figure 2 shows that these human cells generally express at least three ECM inhibitors. The major band of inhibitor activity has an apparent molecular weight of approximately 24-25 kDa, roughly corresponding in molecular weight to ChIMP-3. The major band, human TIMP-3, is most consistent and is found exclusively in the ECM (lanes 4-11) and not in conditioned media (lanes 2 and 3). Its molecular weight is intermediate between that of TIMP-1 (28.5 kDa) and TIMP-2 (21 kDa), both of which were detected in samples of conditioned media (lanes 2 and 3). Human TIMP-3 is expressed at varying levels in different cell lines. There are in addition two minor inhibitors at approximately 29 and 30 kDa, which are designated IMP-a and IMP-b. These two, clearly seen in the ECM of FHs 173We cells (lanes 4 and 5), are less consistent than TIMP-3 and are either smaller (lanes 6 and 7) or larger (lane 10) by approximately 1-2 kDa than the most frequently observed molecular weight of approximately 29 and 30 kDa (lane 4). This variability may be due to

different extents of glycosylation. A fourth inhibitor, which migrates slightly ahead of TIMP-3, is occasionally seen in some samples (see Figure 9).

Figure 3 provides an analysis of conditioned media (left) and matrix (right) of transforming chicken embryo fibroblasts (LA24-CEF) (lane 1) and four mammalian cell lines: SK-N-SH (lane 2), 293 (lane 3), mouse 3T3 cells (lane 5), and rat pheochromocytoma cells derived from an adrenal gland tumor (PC12) (lane 6). Molecular weight standards are in lane 4 (the 30 kDa standard is barely visible).

All four mammalian cells express an inhibitor which co-migrates with ChIMP-2, the chicken equivalent of TIMP-2. Two bands of activity (labelled "A" and "B" to the right of the gel) migrate with an apparent molecular weight of approximately 28-29 kDa, close to the expected mobility for TIMP-1. Significantly, all four mammalian cells express a matrix-specific activity (labelled "TIMP-3" to the right of the gel) whose apparent molecular weight is similar, although slightly larger, than ChIMP-3 (lane 1). It should be noted that mouse TIMP-3 has a deduced molecular weight of 21.7 kDa versus 21.8 kDa for ChIMP-3; however, mammalian TIMP-3 has an apparent molecular weight of approximately 24 kDa on protease/substrate gels.

Figure 4 compares TIMPs present in conditioned media and ECM of mouse 3T3 cells and transforming chicken embryo fibroblasts (LA24-CEF) by protease/substrate gel electrophoresis. The cells were seeded at 2 x 10^6/100 mm dish. The 3T3 cells were grown at 37°C for 36 hours before harvest. LA24-infected cells were cultured at 41°C and transformed by transfer to 35°C for 10 hours before harvest. At this time conditioned media were removed and ECM prepared as described by Staskus et al.

(1991). Lanes 1 and 3 are media samples and lanes 2 and 4 are ECM samples. Molecular weight standards were run in lane 5. This figure shows that the ECM of 3T3 cells contains a major band of activity (marked "TIMP-3"). This protein is clearly different from the media protein C (probably TIMP-2) and the matrix proteins A and B (lane 2), which migrate in positions close to that expected for TIMP-1. The matrix IMPs from 3T3 and also from SK-N-SH cells are not N-glycosylated, as shown by their resistance to PNGase F treatment. These data show that 3T3 and SK-N-SH cells express a matrix-specific IMP which is non-glycosylated.

Example 4: Cloning of Mouse TIMP-3

A mouse cDNA library was screened with a full-length ChIMP-3 probe (Pavloff et al. (1992)) at reduced stringency. Five cDNA clones were obtained. Restriction mapping defined the regions of these cDNAs that cross-hybridized with the ChIMP-3 probe. Four of the clones likely represent overlapping versions of the same sequence; the fifth has an additional EcoRI site towards the 3'-end, suggesting it may result from an incompletely processed or alternatively spliced form.

A 1300 bp fragment which includes the region of homology with ChIMP-3 was sequenced. The sequence contains a single open reading frame that encodes a protein that is 82% identical with mature ChIMP-3 at the amino acid level and 70% identical at the nucleotide sequence level overall, with blocks of identity occurring throughout the predicted coding region. Remarkably, sequences downstream of the open reading frame of the mouse gene were very similar to the 3'-untranslated region of ChIMP-3: one AT-rich stretch of 47 bp is missing only one nucleotide from the corresponding region

of ChIMP-3 and contains a 5'-AATGAAA-3' motif previously noted as being present in human and rabbit TIMP-1. In contrast, the 5'-untranslated sequences of the two genes are poorly conserved.

The 5'- and 3'-untranslated regions of the cDNA are the longest so far described for a member of the TIMP family. The translation start site of the open reading frame is 1 kb from the 5'-terminus of the longest cDNA (clone 7.1) and the 3'-untranslated sequence is an estimated 2.7 kb in length. In contrast, the mature TIMP-1 transcript in mouse and human cells is only 0.9 kb in length.

The extensive similarity of the mouse gene with ChIMP-3 suggests that it is a novel member of the murine TIMP family and should be designated as murine TIMP-3 (mTIMP-3). This designation is substantiated by data confirming that the mouse gene product (1) displays TIMP activity as determined by protease/substrate SDS-PAGE; (2) shows preferential ECM association, a hallmark of ChIMP-3; and (3) is recognized by antisera raised against ChIMP-3.

The nucleotide sequence of mouse TIMP-3 cDNA, aligned with that of ChIMP-3 is shown in Figure 5. In Figure 6, the deduced amino acid sequence of mTIMP-3 is compared with TIMP-1 from bovine, pig, human, rabbit, and mouse TIMP-1 (two sequences); with TIMP-2 from human, mouse and bovine; and with TIMP-3 from chicken. The mature mTIMP-3 protein begins with the "CTCSPS..." as marked.

Example 5: Inducible Expression of Mouse TIMP-3

The expression of mTIMP-3 was compared with that of mTIMP-1 and mTIMP-2 in fibroblasts and adult mouse tissues by Northern blot analysis. A single major

transcript class at around 4.5 kb was found. There is apparently molecular weight heterogeneity because the 3.5 kb TIMP-2 transcript runs on the same blots as a much tighter species. Smaller minor RNAs are also present, but it is unclear whether these are functional mRNAs or breakdown products of the larger form. No transcripts have been observed in the 0.9-1.0 kb range that are characteristic of other murine TIMPs.

Transcription of mTIMP-3 in mouse fibroblasts is highly inducible by stimuli that affect ECM remodelling. Expression of mTIMP-3 was induced in confluent serum-deprived mouse C3H 10T1/2 fibroblasts by exposure to PMA and TGFβ1. The combination of these two agents led to a superinduction of mRNA at early times (i.e., 2-4.5 hr) after exposure. This response is similar to that displayed by mTIMP-1 and distinct from that of mTIMP-2, which is expressed in an essentially constitutive fashion in mouse fibroblasts. Leco et al. (1992). Like TIMP-1, the PMA- and cytokine-mediated induction of mTIMP-3 depends upon ongoing protein synthesis. Maximal induction of mTIMP-3 mRNA(s) occurs from 3-4.5 hr after stimulation, preceding the peak of mTIMP-1 transcript accumulation, which occurs at between 4.5 and 9 hr. Nuclear run-on assays using nuclei isolated from cells exposed to the same treatment combinations showed that increased transcriptional activity presaged the inductions seen in the Northern assays. Transcription of mTIMP-3 had more rapid on/off kinetics, peaking at 3 hr and declining by 4.5 hr, than that of mTIMP-1, which increased continuously through to 4.5 hr after stimulation. The superinduction of both mTIMP-1 and mTIMP-3 by the combination of PMA and TGFβ1 was also clearly demonstrated in the run-on transcription assays.

Another important distinction between the regulation of mTIMP-1 and mTIMP-3 expression was observed when mouse fibroblasts were treated with dexamethasone, either alone or in combination with EGF or PMA. Dexamethasone inhibited TIMP-1 mRNA induction by both EGF and PMA. A similar effect had been observed previously with interleukin-6-induced expression in rat hepatocytes. Roeb et al. (1993). It should be noted that in C3H 10T1/2 fibroblasts, EGF does not activate protein kinase. Edwards and Mahadevan (1992).

In sharp contrast to mTIMP-1, mTIMP-3 expression was stimulated by dexamethasone treatment alone and co-administration with either EGF or PMA augmented the induction seen with these agents separately. These data show that significant differences exist between the regulatory mechanisms that govern mTIMP-1 and mTIMP-3 expression. This is illustrated further by the observation that mTIMP-3 expression is induced equally well by PMA and EGF alone, although for TIMP-1 PMA is clearly the more potent inducer.

Example 6: Purification of Human TIMP-3

Gingival fibroblasts (GF11 and 1292) were obtained as a gift from Dr. D. Richards at UCSF. The following cells were purchased from American Type Culture Collection: transformed primary embryonal kidney (293), neuroblastoma cells (SK-N-SH), normal whole embryo (FHs 173We), cervix epithelioid carcinoma (HeLa S3), colon adenocarcinoma (Caco-2), ileocecal adenocarcinoma (HCT-8) and fibrosarcoma cells (SW 684 and Hs 913T). All human cells were cultured in 100 mm culture dishes (Falcon Labware) at 37°C in a humidified atmosphere of 5% CO_2/95% air with the exception of SW684 cells, which were cultured in a CO_2-free atmosphere. Cells were cultured

in the following media supplemented with 5% fetal bovine serum (FBS): SW 684 cells in Leibovitz L-15; GF11 and 1292 in MEM-α, FHs 173We and Hs 913T in Dulbecco's Modified Eagle's Medium (DMEM) with 4.5 g/l glucose and without sodium pyruvate; HCT in RPMI 1640; Caco-2 in MEM with non-essential amino acids; and HeLa S3 in F12. To eliminate the contribution of serum proteins to the analysis of the ECM, confluent cultures were reseeded 1:2 to 1:3 in their corresponding media without serum and cultured for 48 hours before harvesting ECM as described by Blenis and Hawkes (1983).

Many cell lines (a number of gingival fibroblast lines, adenocarcinomas, a carcinoma and a neuroblastoma) expressed human TIMP-3 at exceedingly low levels, comparable to 10-20% of the amount of ChIMP-3 detected in the chicken system. However, one cell line in particular (FHs 173We whole embryo cells) produced human TIMP-3 at a level which is comparable to that of ChIMP-3 on a per cell basis (40-50 fg/cell) as estimated by protease/substrate SDS-PAGE. However, because human FHs 173We cells are much larger (eight fold larger than chicken cells), the yield of TIMP-3 per culture dish is correspondingly lower.

Normally, it is recommended that the above-listed cells be grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% FCS. However, FCS contains bovine TIMPs. To prevent contamination of quantitative analyses with bovine TIMPs from serum, therefore, cells were grown with as little serum as possible.

FHs 173We cells (normal, human whole embryo - first trimester, ATCC No. HTB 158) were initially seeded under reduced serum conditions (5% FCS) and maintained at 37°C for 5-7 days before passage into serum-free media. Then cells were seeded at a density of 0.35 - 0.45 x

10^6/100 mm culture dish (Falcon) in DMEM (without serum) and maintained at 37°C for 48 hours.

ECM was prepared from the 48 hr cells essentially as described by Blenis and Hawkes (1983). Briefly, the cells were removed from the culture dishes following a 15-20 min incubation in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS), containing 5 mM EGTA, 1 mM phenylmethylsulfonyl fluoride and 1 mg/ml ϵ-amino-n-caproic acid (pH 7.4)(buffer A). After several rinses in PBS containing the protease inhibitors (above) and finally cold (4°C) distilled water, the ECM was solubilized in Laemmli sample buffer (without β-mercaptoethanol).

Because the amount of human TIMP-3 which could be recovered from a single culture dish was considerably lower than ChIMP-3, it was necessary to develop a method to concentrate the human protein. To concentrate human TIMP-3, the ECM samples were reduced by addition of β-mercaptoethanol (final concentration of 5% v/v), heated at 60°C for 10 min and cooled to room temperature. Samples were electrophoresed on sodium dodecyl sulfate-polyacrylamide gels (15%) (SDS-PAGE) (1.5 mm thick), the separating gel having been previously aged at room temperature for at least 24 hr. Sodium thioglycolate was added to the cathode reservoir buffer at a concentration of 0.2 mM. Proteins which electrophoresed as bands in the apparent molecular weight range of 20 to 30 kDa were excised and eluted in 50 mM ammonium bicarbonate, 0.5% SDS and 5 mM dithiothreitol (DTT) at 37°C with occasional shaking for at least 24 hr. The proteins were concentrated by centrifugation of the eluate in microconcentrators with a 30-kDa cut-off (Filtron). In other experiments, the reduced 24 kDa human TIMP-3 protein was concentrated and retained by membranes with

cut-off values of 10 to 50 kDa and partially retained by membranes with cut-off values of 100 to 1000 kDa. This is postulated to be a function of the protein's "stickiness" rather than differences in apparent molecular weight. The concentration of SDS in the retained fraction was monitored by the colorimetric assay of Waite and Wang (1976) Anal. Biochem. 70:279-280. The retained protein was solubilized in Laemmli sample buffer without SDS, to avoid retention of this detergent in the concentrated protein sample. The concentrated protein sample was then re-electrophoresed on a second preparative gel exactly as above.

An alternative concentration procedure includes precipitation of proteins from ammonium bicarbonate elution buffer by the addition of cold (-20°C) methanol (to a final concentration of 90% v/v) and storage overnight at -20°C. Precipitated protein was collected by centrifugation at 10,000 x g for 30 min. The protein was solvated in Laemmli sample buffer containing β-mercaptoethanol (final concentration 5% v/v) and electrophoresed as above.

Proteins resolved on the second preparative gel were transferred electrophoretically to a polyvinylidene difluoride (PVDF - Immobilon-P, Millipore) membrane using 10 mM 3-(-cyclohexylamino)-1-propanesulfonic acid, methanol (10% v/v), pH 11.0, as described by Matsudaira (1987) J. Biol. Chem. 262:10035-10038. Proteins were transferred at 300 mA constant current for two hours. A representative section of the blot was probed with a polyclonal antibody to ChIMP-3 as described in detail below, while the remainder of the blot was stained with Coomassie Blue. The human TIMP-3 was identified on the ChIMP-3 antibody-probed blot as a single band migrating with an apparent molecular weight of approximately 24

kDa. This band was used to locate the protein on the adjacent Coomassie-stained section of the blot.

Excessive manipulation of the protein may have resulted in a large proportion of the protein being N-terminally blocked. Two modifications to the procedure to concentrate the protein without additional electrophoretic manipulation involved: 1) seeding the cells at confluent densities; and 2) increasing the thickness of the resolving gel from 1.5 to 3.0 mm. Electrophoresis was followed by direct transfer to PVDF as described above and localization was obtained with antibody to ChIMP-3.

Example 7: Immunological identification of human TIMP-3 with anti-ChIMP-3 Antibodies

Western blots were obtained using human TIMP-3 isolated and blotted as described above and resolved through two rounds of preparative SDS-PAGE and blotted directly onto polyvinyl difluoridine (PVDF) membrane as described above. Non-specific binding sites on the PVDF were blocked with 1% non-fat, powdered milk (Carnation brand) in Tris buffered saline (TBS), 10 mM Tris HCl, pH 7.4 and 0.9% NaCl, 0.1% normal goat serum for 12 hr at 4°C. The blot was incubated with the primary antibody (e.g., 1/500 dilution of anti-ChIMP-3 in TBS containing 1% BSA) for 12 hr at 4°C and rinsed extensively with TBS (see below). It was then incubated with secondary antibody, goat anti-rabbit IgG, conjugated to alkaline phosphatase (Vector Laboratories) (1/1000 dilution in TBS containing 1% BSA) for 2 hr at room temperature. After further rinsing in TBS (see below), the immunoblot was developed by adding substrate for alkaline phosphatase (Kit II, Vector Laboratories). The blots were rinsed in different TBS solutions in the following order: TBS

(once); TBS, 0.1% Tween 20, 0.5 M NaCl (once); TBS, 0.1% Tween 20 (twice); and TBS (twice).

In Figure 7, Western blots of reduced proteins from human ECM and CM were probed with polyclonal antibodies against human TIMP-1, ChIMP-3 and human TIMP-2. Conditioned media (CM) from FHs 173We was dialyzed overnight against 0.01% w/v SDS using Spectra/Por-1 tubing (6,000-8,000 molecular weight cutoff, Spectrum Medical Industries), then concentrated 150-fold by low-speed centrifugation under vacuum in a Speed-Vac (Savant Instruments). The concentrated CM and solubilized ECM from FHs 173We cells were reduced in β-mercaptoethanol (final concentration 5% v/v) and electrophoresed on a 1.5 mm thick SDS-15% polyacrylamide slab gel. Electrophoretic transfer to PVDF membrane (Immobilon-P, Millipore) was performed using 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) buffer, pH 11.0 containing methanol (10% v/v) according to the method described by Matsudaira (1987) J. Biol. Chem. 262:10035-10038. Transfer was accomplished at 300 mA for 2 h. Non-specific binding sites on the membrane were blocked with 1% w/v nonfat dry milk powder (Carnation) in TBS, 10 mM Tris-Cl, pH 7.4, 0.9% NaCl, 0.1% normal goat serum for 12 h at 4°C. The blot was then incubated with the primary antibodies (diluted 1:500 in TBS containing 1% w/v BSA) either overnight at 4°C or at room temperature for at least 8 h. The blot was then rinsed extensively in the following sequence: one rinse with TBS, one rinse with TBS containing 0.1% v/v Tween 20 and 0.5 M NaCl, two rinses with TBS with 0.1% v/v Tween 20 and two rinses with TBS. The blots were then incubated with the secondary antibody, goat anti-rabbit IgG conjugated to alkaline phosphatase (BioRad Laboratories) (diluted 1:3000 in TBS containing 1% w/v BSA at room temperature

for 2-4 h and rinsed extensively as described above. The blots were developed using an alkaline phosphatase conjugate substrate kit (Kit II, Vector Laboratories).

In Figure 7, lanes 2, 3, and 5 contain ECM from human FHs 173We cells (approximately 2.4 x 10^6 cells). Lanes 1 and 6 contain dialyzed and concentrated CM (equivalent to 60 μl CM) from FHs 173We cells. Lane 4 contains ECM from CEF cells (approximately 4 x 10^6 cells). The data in this figure were derived from three different gels and therefore the mobilities of the proteins cannot be compared directly. The apparent molecular weights for the reduced proteins recognized by each antibody were determined by reference to molecular weight standards on each individual gel (not shown).

These Western blots show that anti-ChIMP-3 recognizes a protein of the appropriate weight for ChIMP-3 in CEF cells. In the ECM of human cells (lane 3), anti-ChIMP-3 recognizes a major protein of approximately 24 kDa, intermediate in molecular weight between TIMP-1 and TIMP-2 and migrating slightly more slowly than the control ChIMP-3 in lane 4, together with a minor band of approximately 29 kDa. In contrast, anti-human TIMP-1 (lane 2) and anti-human TIMP-2 (lane 5) do not recognize any proteins in the ECM samples, although they recognize authentic TIMP-1 (lane 1) and TIMP-2 (lane 6) in samples of conditioned media from the whole embryo cells. Under similar conditions, polyclonal anti-ChIMP-3 recognizes human TIMP-3 but not human TIMP-1 or TIMP-2.

Human TIMP-3 represents a relatively small percentage of ECM proteins in all human cell lines that have been screened. This is in contrast to ChIMP-3, which is a major component of the ECM of chicken embryo fibroblasts during the early stages of transformation. As a result, it was not possible to identify the human

TIMP-3 among other proteins on polyacrylamide gels stained with silver or Coomassie Blue or on autoradiograms of [^{35}S]methionine labeled proteins separated by SDS-PAGE. The only assays that have allowed identification of the protein are protease/substrate gel electrophoresis and Western blot analysis using anti-ChIMP-3 antibodies as probes. The former assay is extremely sensitive and allows the detection of as little as 5 fmoles of ChIMP-3; the latter assay, together with estimates of molecular weight, provides unambiguous identification of human TIMP-3.

Figure 8 shows a Western blot of human TIMP-3 in a reduced and unreduced state showing the effect of β-mercaptoethanol on the electrophoretic migration of human TIMP-3 on SDS polyacrylamide gels. Samples of ECM were electrophoresed on SDS polyacrylamide gels. The proteins were transferred electrophoretically to PVDF membrane and probed with an antibody to the NH_2-terminus of ChIMP-3. Lanes 1-4 contain ECM from approximately 2.4 x 10^6 FHs 173We cells; lane 6 contains ECM from approximately 4 x 10^6 CEF; lane 5 contains sample loading buffer only. (+) indicates addition of β-mercaptoethanol to a final concentration of 5% v/v; (-) indicates no addition of β-mercaptoethanol. Human TIMP-3, when reduced with β-mercaptoethanol, runs more slowly than the unreduced control and at approximately the same apparent molecular weight as reduced ChIMP-3 (in lane 6, reduced ChIMP-a clearly resolves from reduced ChIMP-3). In lane 2, where there is a gradient of reducing agent between lane 1 (no β-mercaptoethanol) and lanes 3 and 4 (with β-mercaptoethanol), the protein shows an altered pattern of mobility that is reminiscent of that observed for ChIMP-3. Staskus et al. (1991).

Example 8: The Bulk of Human TIMP-3 is not N-Glycosylated

ECM was prepared from human neuroblastoma (SK-N-SH) cells that had been cultured with 5% serum and then maintained for 24 hours in serum-free media. The ECM was solubilized in Laemmli buffer without reducing agent and dialyzed extensively against 0.2 M sodium phosphate, pH 8.0, containing 10 mM EDTA, 0.05% w/v SDS at 4°C using Spectro/Por-3 tubing (3,500 molecular weight cut-off, Spectrum Medical Industries). The sample volumes were reduced by coating the dialysis tubing with carboxymethylcellulose (Aquacide I, Calbiochem) to absorb water. To 38 μl of sample (corresponding to ECM prepared from 1.6 of a 100 mm culture dish) was added 2 μl octyl glucoside (10% w/v) in the phosphate buffer, for a final glucoside concentration of 0.5% w/v. Recombinant N-glycosidase-F (N-glycanase, Genzyme Corp.), which cleaves N-linked oligosaccharides from a protein backbone, was then added in a volume of 2 μl (0.5 units); and the sample was incubated at 37°C for 8 hours. Aliquots equivalent to 1/10th of the material isolated from one culture dish (100 mm), as well as samples not treated with N-glycosidase-F, were electrophoresed on a protease/substrate gel.

As shown in Figure 9, the bulk of human TIMP-3 is not N-glycosylated. IMP-a and IMP-b proteins clearly changed in mobility following incubation with N-glycosidase-F, but there was no apparent change in the mobility of the major band of human TIMP-3. ChIMP-3 is similarly not glycosylated. Pavloff et al. (1992). It is assumed that the deglycosylated IMP-a and IMP-b proteins migrate with molecular weights which are similar enough to TIMP-3 to be masked by the broad band of TIMP-3's inhibitor activity, as no additional bands of

activity are apparent on the gel. IMP-a and/or IMP-b may be an N-glycosylated form of TIMP-3.

Example 9: Cell Detachment Assay

First, in order to prepare culture dishes, uncoated plastic dishes are coated with the control protein histone H1 (5 μg/dish), electrophoretically purified human TIMP-3, or a "mock" sample isolated from a blank gel under identical conditions. Uncoated dishes are used as an additional control. Proteins are dissolved in 10 mM sodium phosphate buffer, pH 7.0/9 M urea and sterilized by passage through 0.2 μm membranes (Acrodisc, Gelman). Protein solutions (0.15 ml) are distributed to 60-mm culture dishes and diluted 10-fold by addition of Tris diluent (137 mM NaCl/5 mM KCl/5 mM Na_2HPO_4/25 mM Tris base, pH 7.2) and incubated at 41°C for four days. The solution is removed by aspiration and the surface rinsed with the appropriate medium before seeding cells. Binding of human TIMP-3 to the dishes can be monitored by use of ^{35}S-labeled protein. After 12 hr, 2 days and 4 days, the percentage of binding is determined (μg per 60-mm dish).

LA24-infected chicken embryo fibroblast (CEF) cells may be used. Preferably, however, transforming mammalian cells, particularly human cells, are used and are cultured as described above (including the use of DMEM without serum). If CEF cells are employed, the cells (1 x 10^6 per 60 mm dish in 5 ml of medium 2-2-1+ (Yang and Hawkes (1992)) are incubated at the nonpermissive temperature (41°C) for 6 hr to promote attachment to the culture dishes. Controls include untreated plastic dishes, dishes coated with histone and dishes treated with a sample eluted from a blank gel. Then half of the dishes are transferred to the permissive

temperature (35°C) for 16 hr to allow development of the transformed phenotype.

Duplicate cultures are then assayed for cell detachment. The assay for cell detachment from the ECM is a modification of published procedures. Johnson and Pastan (1972) Nature 236:247-249; and Shields and Pollock (1974) Cell 3:31-38. The medium is decanted and the cell monolayer is washed twice with Ca^{2+}-, Mg^{2+}-free phosphate-buffered saline (pH 7.4) and incubated in the same buffer containing 5 mM EGTA (2 ml per 60 mm dish) for 10 min at 41°C. The dishes are agitated on a rotary shaker (Braun Thermonix Shaker 1460) at 120 rpm for 5 min at room temperature. EGTA-released cells are removed and the remaining cells are detached by trypsin (0.05% wt/vol, 41°, 15 min). The number of cells in each sample is determined by counting in a Coulter Counter. Cells detached by EGTA treatment are expressed as a percentage of the total cell number.

Example 10: Cell Proliferation Assay

LA24-infected CEF cells, or preferably, mammalian cells, and particularly human cells such as FHs 173We may be used, as for Example 9. If CEF cells are used, the cells are seeded at 2 x 10^5 per 60-mm dishes in low serum medium (2-0-0.1+), cultured at 35°C or 41°C, detached with trypsin (as above), and counted at 24-hr intervals. The population doubling time is determined from a graph of logarithm of cell number versus time in culture. Values are calculated as averages of triplicate determinations.

Example 11: Expression of Human TIMP-3 mRNA in Human Tissues

ChIMP-3 cDNA was used to probe the mRNA of a large number of cultured mammalian cells. Evidence was found of the expression of TIMP-3 in rat, mouse, monkey, and human cells.

ChIMP-3 cDNA (P483, including the coding region) was random primed with ^{32}P and used to probe RNA dot blots. Hybridization was observed with RNA from human endometrial adenocarcinoma cells (HEC-1-B), human newborn foreskin cells (Hs27), human breast adenocarcinoma cells (MCF-7), two lines of mutant human fibroblasts (GM03123 and GM00110B), as well as African green monkey kidney cells (CV-1).

Northern blots of RNA from chicken cells (CEF) probed with a ^{32}P-labeled PCR probe from the ChIMP-3 cDNA (P483) showed transcripts at approximately 4500-5500 nt, 2300-2900 nt, and 1000 nt. Preliminary results from the probing of northern blot of human fetal heart, lung, and kidney cells (Clontech) with a PCR probe prepared from the human cDNA corresponding to amino acids 88-109 of ChIMP-3, the cDNA being prepared as described in Example 13 below, revealed hybridizing species at approximately 4500-5500 nt and 2500 nt. It is not yet known whether a longer exposure will reveal a smaller transcript corresponding to the 1000 nt chicken transcript.

Example 12: Purification, Protein Sequencing, and Amino Acid Analysis of Human TIMP-3

ECM was prepared from confluent FHs 173We cells using a total of 45 culture dishes (100 mm). The material was solubilized in Laemmli sample buffer containing β-mercaptoethanol (final concentration 5% v/v) and electrophoresed in three lanes through a preparative SDS-polyacrylamide gel (12% acrylamide, 3 mm thick) using the gel/buffer system described by Laemmli (1970) Nature 227:680-685. In order to minimize the possibility of NH_2-blocking during electrophoresis, the separating gel was polymerized at room temperature for 48 h and the free radical scavenger sodium thioglycolate was included in the cathode buffer during electrophoresis at a final concentration of 0.1 mM according to the method described by Hunkapiller et al. (1983) Met. Enzymol. 91:227-236. Furthermore, all solutions for electrophoresis were freshly prepared from previously unopened containers of electrophoresis grade reagents (Bio-Rad Laboratories). Following electrophoresis, proteins were transferred (240 mA, 12 h) to a PVDF membrane (Pro-Blott™, Applied Biosystems) in 10 mM CAPS buffer, pH 11.0, containing methanol (10% v/v) according to the method described by Matsudaira (1987). The blot was rinsed extensively with double distilled water and a section of the blot containing a small aliquot of ECM from FHs 173We cells was excised and probed with the polyclonal antibody to the ChIMP-3 peptide, as described above, to locate TIMP-3. The remainder of the blot was stained for one minute with Coomassie Blue R-250 in acetic acid/methanol/water (1:50:49), destained in methanol/water (50:50), rinsed extensively with water, dried, and stored at -20°C. The antibody-probed blot was then aligned with the Coomassie-stained blot and used to locate the TIMP-3 bands. These

were excised and half of the sample was subjected to N-terminal microsequencing and the remainder reserved for amino acid composition analysis using the Pico-Tag method (Waters Associates) according to the method described by Bidlingmeyer et al. (1984) J. Chromatogr. 336:93-104.

The sequencing was performed by repetitive Edman degradation using an Applied Biosystems Model 473 A protein sequencer utilizing standard chemistry and programs. Hunkapiller et al. (1983) Met. Enzymol. 91:399-413. The phenylthiohydantoin amino acids were identified by HPLC on an Applied Biosystems 120A PTH analyzer (Hunkapiller et al. (1985) PTH Amino Acid Analysis, User Bulletin Number 14, Applied Biosystems, Foster City, CA).

Amino acid sequence analysis of purified human TIMP-3 indicates, for those residues determined, that its NH_2-terminus is identical to that of ChIMP-3 (Pavloff et al. (1992)) and the recently cloned mouse TIMP-3 (Leco, K.J., Khokha, R., Pavloff, N., Hawkes, S.P., and Edwards, D.R. (1993), submitted) (Figure 10). Of those positions where the chicken and mouse sequences differ (valine/serine at position 4 and isoleucine/serine at position 6 for ChIMP-3/mTIMP-3), no assignments could be made. Cysteines at positions 1, 3, and 13, which are highly conserved in all TIMPs, could not be determined without prior derivatization of the protein with 4-vinyl pyridine. In Figure 10, hTIMP-3 is human TIMP-3; ChIMP-3 is chicken TIMP-3 (Pavloff et al. (1992)); and mTIMP-3 is mouse TIMP-3 (Leco et al. (1993)). $TIM\text{-}1_{con}$ is a consensus sequence for TIMP-1 from human (Docherty et al. (1985); Gasson et al. (1985); and Carmichael et al. (1986)); murine (Edwards et al. (1986); Gewert et al. (1987); and Johnson et al. (1987)); rabbit (Horowitz et al. (1989)); bovine (Freudenstein et al. (1990)); porcine

(Tanaka et al. (1992)); and rat (Roswit et al. (1992)) tissues. TIMP-2_{con} is a consensus sequence for TIMP-2 from human (Boone et al. (1990); and Stetler-Stevenson et al. (1990)); bovine (Boone et al. (1990)); murine (Leco et al. (1992); and Shimizu et al. (1992)); rat (Roswit et al. (1992)); and chicken (Staskus et al. (1993)) sources. In Figure 10, a dash indicates residues that were not determined. Bold letters indicate residues conserved among all TIMPs. Lower-case letters indicate residues which are variable among species for a particular TIMP. Underlined amino acids (residue 10) indicate possible unique conservation within each member of the TIMP family. Additional letters under the lower-case letters in the consensus sequences indicate different amino acids for other species.

The amino acid composition of human TIMP-3 is similar to ChIMP-3. The major discrepancy is the high glycine content in the chicken protein (23-34 residues compared to 17 in human TIMP-3). This was previously attributed to contamination with this amino acid from buffers during purification. Staskus et al. (1991). Indeed, the deduced amino acid composition from ChIMP-3 cDNA analysis indicates 15 glycine residues from a total of 188 amino acids in the mature protein. Pavloff et al. (1992).

In other experiments, the TIMP-3 protein, which was identified on PVDF with anti-ChIMP-3 antibody, was eluted in 50 mM Tris-HCl buffer, pH 9.0, containing 2% SDS/1% Triton X-100 and analyzed by protease/substrate gel electrophoresis. A single metalloproteinase inhibitory activity was detected as a band which co-migrated with human TIMP-3 present in a sample of total ECM proteins.

Example 13: Cloning and Sequencing of Human TIMP-3

Cell culture and tissues. Human concanavalin A activated peripheral blood lymphocytes were obtained in the laboratory. A human hepatoma cell line, U 937 (ATCC 1593), and a human B lymphoblast cell line, WI-L2-729HF2 (ATCC CRL8062), were obtained from the American Type Culture Collection. Cells were grown in RPMI medium containing 10% FBS in 5% CO_2.

RNA Isolation. RNA was isolated by the guanidinium thiocyanate method according to the method described by Chirgwin et al. (1979) Biochem. 18:5294-5299 with modifications according to the method described by Freeman et al. (1983) Proc. Natl. Acad. Sci. USA 80:4094-4098. Poly(A)$^+$ RNA was purified by a single fractionation over oligo(dT)-cellulose according to the method described by Aviv and Leder (1972) Proc. Natl. Acad. Sci. USA 69:1408-1412. Total RNA from human heart was obtained from Clontech (Palo Alto, CA). First strand cDNA was synthesized as described by Zapf et al. (1990) Biochem. Biophys. Res. Commun. 158:1187-1194.

cDNA library. A λgt10 human heart 5' stretch cDNA library was purchased from Clontech. A human placenta cDNA library was a generous gift from Dr. N. Lomri at UCSF, San Francisco, CA.

Oligonucleotide Synthesis. Oligonucleotide primers for PCR and probes were synthesized by the phosphoramidite method with an Applied Biosystems model 394 automated DNA synthesizer and purified by PAGE and desalted on Sep-Pak (Millipore Corp.) using standard protocols.

Primer TIMP-4 was designed to bind to the noncoding strand of TIMP-3 cDNA corresponding to amino acids 82-87 of ChIMP-3 (YIYTEA, Pavloff et al. (1992)), with amino acid 82 substituted by Y or F to cover other

possible TIMP sequences and amino acid 84 substituted by Y (chicken sequence) or H (mouse sequence). This sequence was chosen for the relatively small codon degeneracy and for the least possible sequence similarity to other TIMPs. The sequence of the 26-base primer consists of 17 bases specific for TIMP-3, a 6-base EcoRI restriction site and three extra bases at the 5′ end (underlined).

Primer TIMP-4:
5′-GACGAATTCT(T,A)(T,C)AT(A,C,T)(C,T)A(C,T)ACIGA(A,G)GC-3

Primer TIMP-5 was designed to bind to the coding strand corresponding to amino acids 110-115 of ChIMP-3 (YEGKVY, Pavloff et al. (1992)), with amino acid 114 substituted by M (mouse TIMP-3) and amino acid 115 substituted by Y or H to cover other possible TIMP-3 sequences. This sequence was chosen for the relatively small codon degeneracy and for the least possible sequence similarity to other TIMPs. The sequence of the 26-base primer consists of 17 bases specific for TIMP-3, a 6-base HindIII restriction site and three extra bases at the 5′ end (underlined).

Primer TIMP-5:
5′-GACAAGCTT(A,G)T(A,G)CAT(C,T)TTICC(C,T)TC(A,G)TA-3′

Primer TIMP-3-9-3′ was designed on the basis of partial sequence information on human TIMP-3. It was used to clone the 3′ end of TIMP-3 and included a 6-base HindIII restriction site and six extra bases at the 5′ end (underlined).

Primer TIMP-3-9-3':
5'-AGATCTAAGCTTTCCGAGAGTCTCTGTGGCC-3'

One additional oligonucleotide primer 29 (26-mers) was designed to clone the 3' end of human TIMP-3. Primer 29 was designed to bind to the noncoding strand corresponding to 18 bases in the 3'-noncoding region of ChIMP-3 which is identical in mouse TIMP-3 (nucleotides 831-851, Pavloff et al. (1992)). This sequence was chosen because the sequence AATGAAA (nucleotides 840-846 in ChIMP-3), which we noted was a variant of a poly(A) signal, is also present at the 3' end of human (Docherty et al. (1985); Carmichael et al. (1986)); and rabbit (Horowitz et al. (1989)) TIMP-1 cDNA and precedes the putative polyadenylation consensus sequence signal, AATAAA. The sequence of the 26-base primer consists of 18 bases specific for ChIMP-3, a 6-base BamHI restriction site and two extra bases at the 5' end (underlined).

Primer 29:
5'-GCGGATCCACTAATTTCATTGTCATC-3'

Primer TIMP-3-1-67 was designed on the basis of partial sequence of human TIMP-3 and was used as a probe to screen a λgt10 human heart cDNA library purchased from Clontech.

Primer TIMP-3-1-67:
5'-GACGCGACCTGTCAGCAGGTACTGGTACTTGTTGACCTCCAGCTTAAGGCC ACAGAGACTCTCGGAA-3'

PCR (Polymerase Chain Reaction) and Cloning.
The DNA templates used in PCR were the following: Human

concanavalin A-activated peripheral blood lymphocytes cDNA, U 937 cDNA, WI-L2-729HF2 cDNA, cDNA from human heart, human placenta cDNA library, and genomic DNA from Clontech. The PCR was run as described by Perkin Elmer Cetus using Ampliwax. Initially, primer TIMP-4 was used along with primer TIMP-5. The PCR was run in a DNA thermal cycler (Perkin Elmer Cetus) for 35 cycles. The first 10 cycles consisted of heating at 94°C for 1 min., and annealing at 36°C for 2 min. The last 25 cycles consisted of heating at 94°C for 1 min., annealing at 55°C for 1 min., and polymerization at 72°C for 1 min.

This reaction consistently yielded a single 114 bp product (P114) detected on a 7% acrylamide gel representing a partial human TIMP-3 cDNA present in all the DNA tested. After treatment with the Klenow fragment, P114 obtained from all these PCR products was digested with EcoRI and HindIII, fractionated on a 7% acrylamide gel and cloned into M13 mp19. Three independent subclones were selected.

After sequence analysis of P114, a specific primer (TIMP-3-9-3′) was designed to determine the 3′-end sequence of the cDNA. This primer and primer 29 (described above) were used to amplify cDNA from the λgt10 library. The PCR was run for 30 cycles, each consisting of heating at 94°C for 1 min., annealing at 55°C for 2 min. and polymerization at 72°C for 3 min. After treatment with the Klenow enzyme, the resulting single 450 bp fragment (P450) was cloned in pCRScript™SK(+) (Stratagene).

A ^{32}P-labeled probe, TIMP-3-1-67, was generated by the kinase reaction for subsequent screening of the λgt10 library. Unincorporated nucleotides were removed on a Sephadex G-25 column (Boehringer Mannheim Corporation).

Screening the cDNA Library. Approximately $3x10^5$ phage were grown on six 150 mm plates, lifted in duplicate on a supported nitrocellulose transfer membrane (BAS-NC, Schleicher & Schuell) and hybridized to the partial cDNA probe, ^{32}P-labeled TIMP-3-1-67, described above. Hybridizations were performed overnight at 42°C in 2X Denhardt's solution, 6X SSC (SSC is 15 mM sodium citrate, 150 mM NaCl), 0.4% SDS (w/v), 500 µg/ml salmon sperm DNA. The filters were washed in 2X SSC containing 0.05% SDS (w/v) at 60°C. Positively hybridized plaques were subject to DNA sequencing.

Standard protocols for cDNA library screening, lambda phage purification, agarose gel and acrylamide gel electrophoresis and plasmid cloning were employed (Maniatis et al. (1982) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

DNA Sequencing. Double-stranded DNA cloned into pCR-Script™SK(+) and single-stranded DNA cloned into M13mp19 were sequenced by the dideoxy terminator method (Sanger et al. (1977) Proc. Natl. Acad. USA 74:3463-3467) using sequencing kits purchased from United States Biochemicals (Sequenase version 2.0). Each cDNA subclone was sequenced using an M13 universal primer and a reverse sequencing primer.

Figure 11 shows nucleotide sequences of human TIMP-3 cDNA corresponding to 18 amino acids of the signal peptide and the entire (188 amino acids) mature protein. The nucleotide sequence includes additional sequences from the 3'-noncoding region.

The human TIMP-3 sequences obtained (Figure 11) have approximately 82% nucleotide sequence identity and 91% amino acid sequence identity with ChIMP-3 over this region. There was little nucleotide sequence similarity

between the human TIMP-3 and ChIMP-3 genes in the 3'-noncoding region sequenced initially.

Additional sequencing has been performed essentially as described above. Primer TIMP-3-9-3' designed on the basis of partial sequence information on human TIMP-3 and primer 29 designed to bind to the noncoding strand of TIMP-3 cDNA were used to clone the 3' end of TIMP-3 in a PCR on the library. A single 450-base pair product (P450) resulted. This was cloned into pCR-SCRIPT™SK(+) (Stratagene). One independent clone was sequenced. 450 nucleotides specific for human TIMP-3 were identified.

A comparison of the nucleotide sequence of ChIMP-3 (amino acids 88-212) with the 3' end coding sequence of human TIMP-3 indicates that 292 nucleotides are identical out of the 378 total. This represents 77% nucleotide sequence identity. A comparison of the 125 deduced amino acid residues of human TIMP-3 with the deduced amino acid sequence of ChIMP-3 indicates that 102 amino acid residues are conserved. This represents about 82% amino acid sequence identity. There was not much homology in the 75 noncoding nucleotides.

The complete sequence of the mature region of human TIMP-3 has been determined by screening the human cDNA library using ^{32}P-labeled TIMP-1-67 as a probe. Seven positive clones were isolated and are being purified. Obtaining a genomic sequence for human TIMP-3 is readily accomplished by using the human TIMP-3 cDNAs as probes or by designing PCR primers using sequences from the cDNA which are likely to distinguish TIMP-3 from TIMP-1 and -2, as discussed above, particularly from the noncoding regions of the gene.

All patents and patent applications cited in this specification are incorporated herein by reference

to the same extent as if each was specifically and individually indicated to be incorporated by reference.

This invention has been detailed both by example and by direct description. It should be apparent that one having ordinary skill in this art would be able to surmise equivalents to the invention as described in the claims which follow but which would be within the spirit of the description above. Those equivalents are to be included within the scope of this invention.

WHAT IS CLAIMED IS:

1. An isolated human TIMP-3 polypeptide.

2. The polypeptide according to claim 1 wherein the polypeptide is obtained by expression of recombinant DNA encoding the protein.

3. The polypeptide according to claim 1 wherein the polypeptide is obtained from human extracellular matrix.

4. The polypeptide according to claim 1 having the amino acid sequence in Figure 11.

5. The polypeptide according to claim 2 encoded by the nucleic acid sequence in Figure 11.

6. An isolated nucleic acid sequence encoding human TIMP-3.

7. The nucleic acid sequence according to claim 6 having the sequence shown in Figure 11.

8. The nucleic acid sequence according to claim 7 further comprising a functional signal peptide.

9. A composition comprising antibodies specific for human TIMP-3 polypeptide.

10. The composition of claim 9 wherein the antibodies are monoclonal or polyclonal.

11. A method of detecting the presence of human TIMP-3 polypeptide in a sample, said method comprising the steps of:

a) contacting said sample with an antibody specific for human TIMP-3, under conditions suitable for the formation of a human TIMP-3-antibody complex; and

b) detecting the presence of said complex.

12. A method of detecting elevated levels of human TIMP-3 polypeptide in a test sample, said method comprising the steps of:

a) contacting said test sample with an antibody specific for human TIMP-3, under conditions suitable for the formation of a human TIMP-3-antibody complex;

b) contacting a control sample with said antibody, under conditions suitable for human TIMP-3-antibody complex formation;

c) detecting the level of human TIMP-3-antibody complex formation in said control sample and in said test sample; and

d) comparing the levels of complex formation in said control sample and said test sample.

13. A pharmaceutical composition comprising the human TIMP-3 polypeptide of claim 1 and a pharmaceutically acceptable excipient therefor.

14. A pharmaceutical composition comprising a human TIMP-3 polypeptide according to claim 2 and a pharmaceutically acceptable excipient therefor.

15. A pharmaceutical composition comprising a human TIMP-3 polypeptide according to claim 3 and a pharmaceutically acceptable excipient therefor.

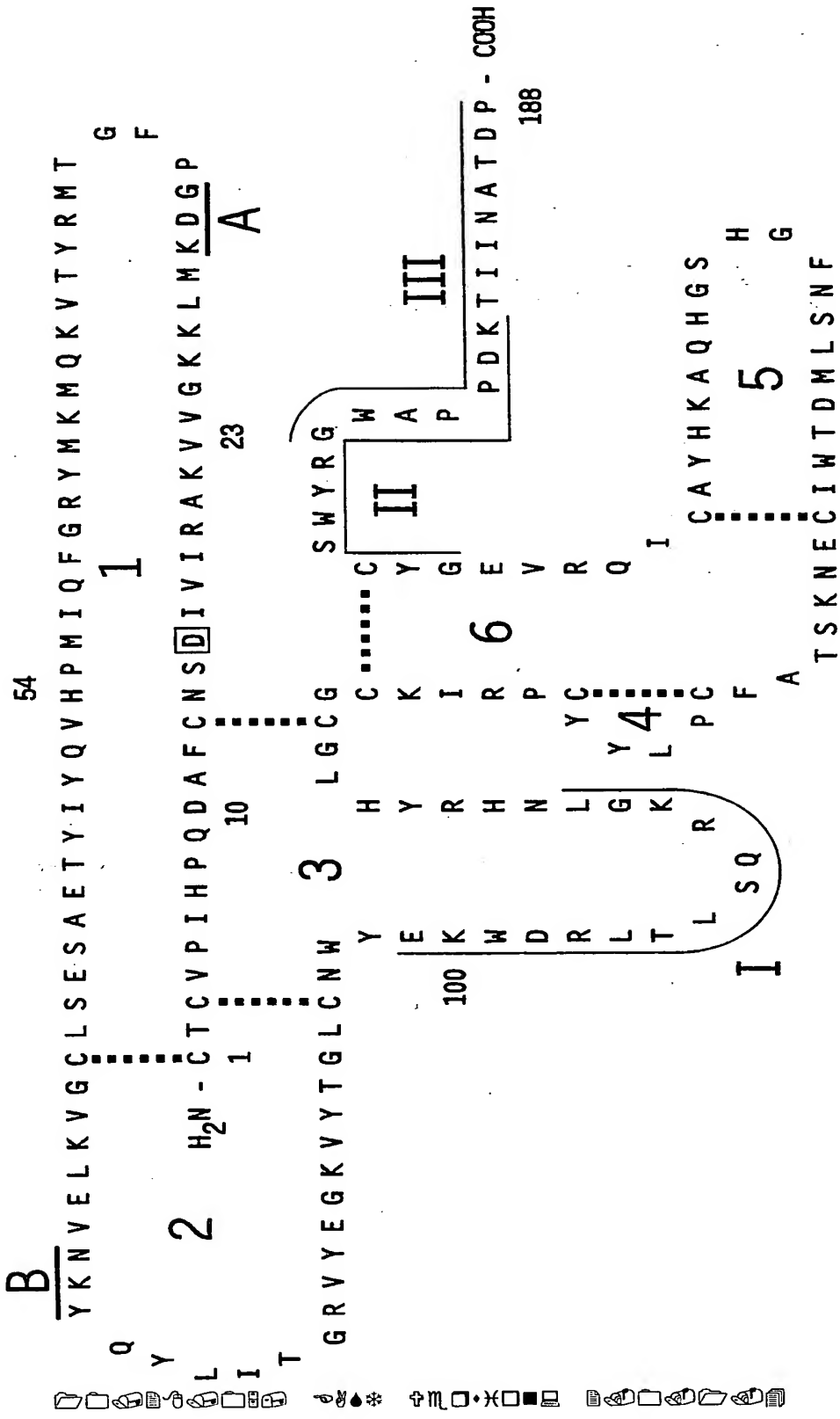
B
A
1
2
3
4
5
6
I
II
III
H_2N - C T C V P I H P Q D A F C N S D I V I R A K V G K K L M K D G P
Y K N V E L K V G C L S E S A E T Y I Y Q V H P M I Q F G R Y M K M Q K V T Y R M T
G R V Y E G K V Y T G L C N W L G C G S W Y R G C C Y G E V R Q I C A Y H K A Q H G S
T S K N E C I W T D M L S N F
P D K T I I N A T D P - COOH
10
23
54
100
188

FIG. 1

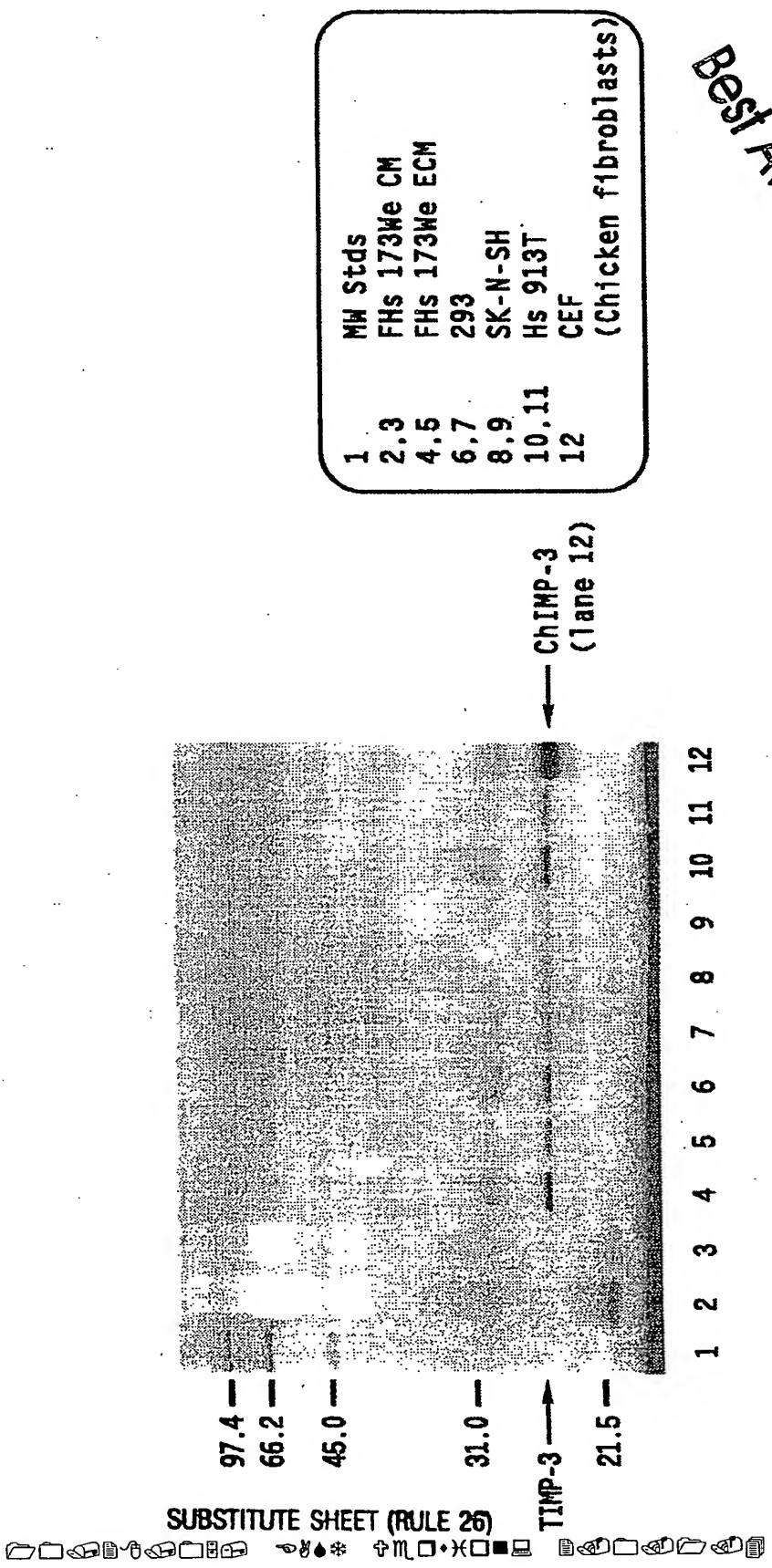

FIG. 2

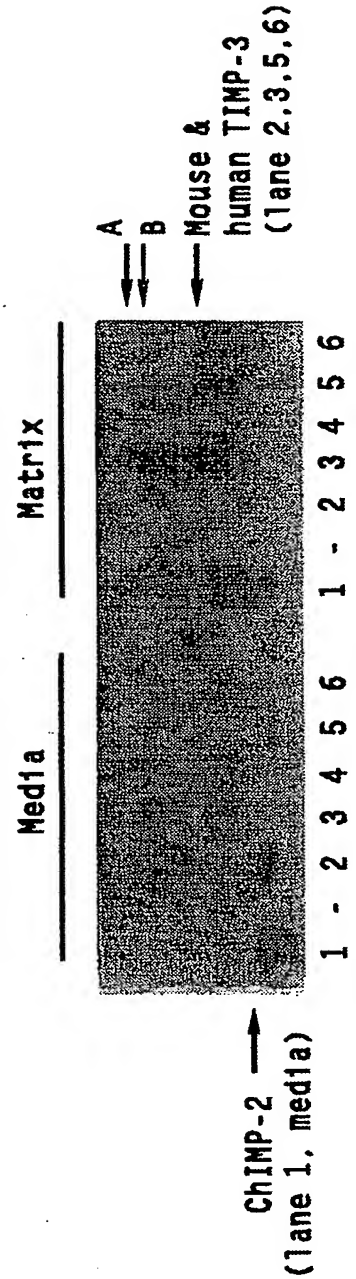

FIG. 3

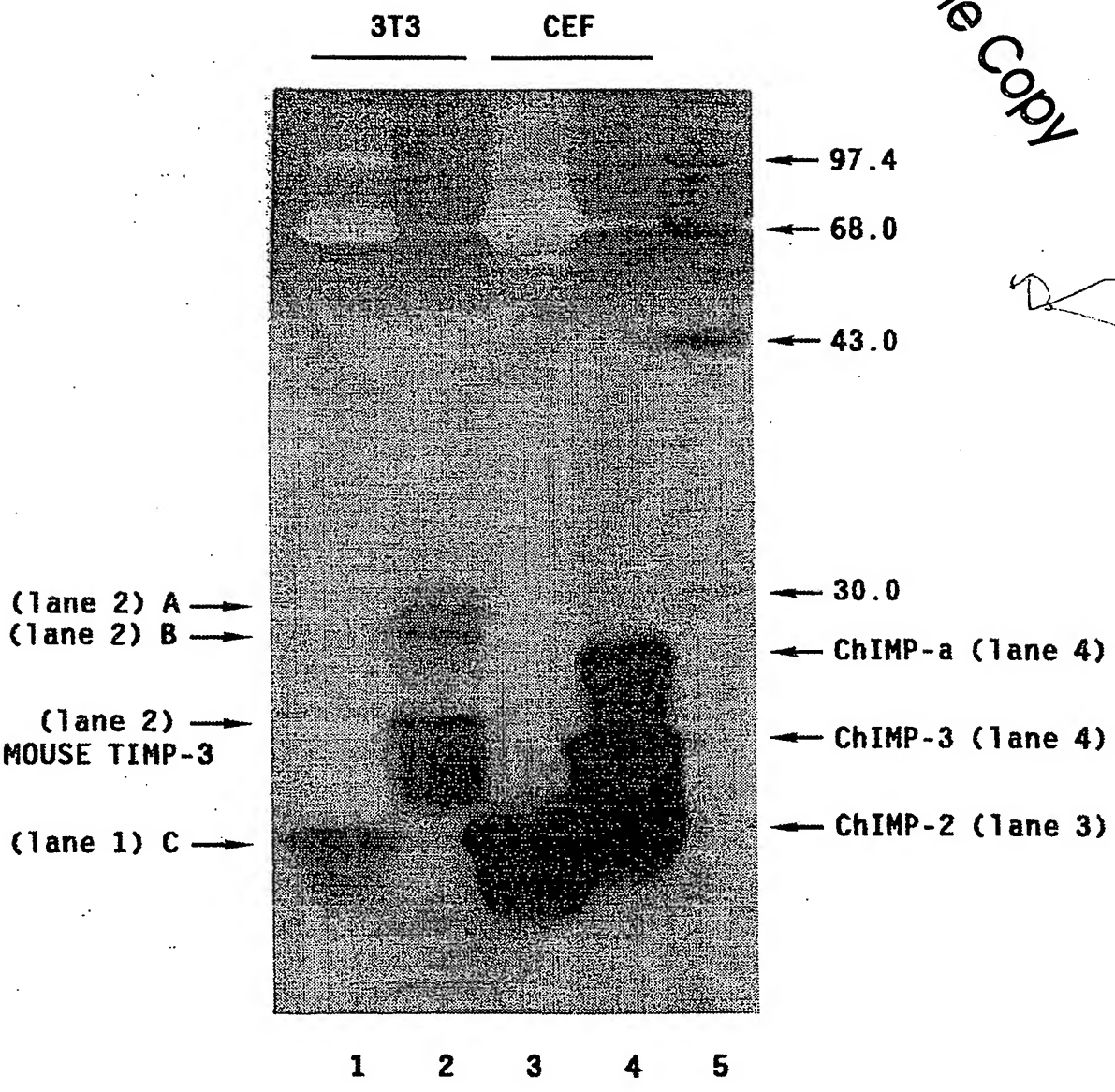

FIG. 4

FIG. 5(A)

```
TIMP-3   151 GCCGGGCTACTTGGAAGGCACTTCCCCGGAGCTCATCGTTGCCCACCGTG 200
ChIMP-3    1 ..........................................CCC 3
         201 CACAGTGCCCGGTTAAACCCAGCGAGTGAGCTCGGACTGTAGCATCAGCG 250
           4 GAGAGAGAGGCGGTGTGAGGAGGGAGCGAGCGAGCAGCGAACAGGCGAGG 53
         251 CTACGCTCGGCAACTTTGAAGAAAAGAGCGGCAGTCCCCGCAGCGGACCA 300
          54 CTCGAGTTAG..GCGAACAGAACAGCGGCTGCAGCTCGAAGCGCACCCCG 101
                        START
         301 CAACAGCTACCATGACTCCCTGGCTTGG...GCTTGTCGTGCTCCTGAGC 347
         102 GGGCAGGCAGCATGACGGCGTGGCTCGGCTTCCTCGCCGTGTTCCTGTGC 151
         348 TGCTGGAGCCTTGGGCACTGGGGCGCGGAAGCGTGCACATGCTCTCCCAG 397
         152 AGCTGGAGCCTGCGGGACCTGGTGGCGGAGGCGTGCACTTGCGTCCCCAT 201
         398 CCATCCCCAGGATGCCTTCTGCAACTCCGACATCGTGATCCGGGCCAAAC 447
         202 CCACCCGCAGGACGCGTTCTGCAACTCCGACATCGTGATCCGTGCTAAAG 251
```

FIG. 5(B)

```
448 TGGTGGGAAAGAAGCTGGTGAAGGAGGGGCCCTTTGGCACTCTGGTCTAC 497
    ||||||||||||||||||||||||||||||||||||||||||||||||||
252 TTGTGGGGAAGAAGCTCATGAAAGATGGACCATTTGGAACAATGCGATAC 301

498 ACTATTAAGCAGATGAAGATGTACCGAGGCTTCAGTAAGATGCCCCACGT 547
    ||||||||||||||||||||||||||||||||||||||||||||||||||
302 ACAGTCAAGCAGATGAAGATGTACAGGGGCTTCCAGATAATGCCACACGT 351

548 GCAGTACATTCACACGGAAGCCTCTGAAAGTCTTTGTGGCCTCAAGCTAG 597
    ||||||||||||||||||||||||||||||||||||||||||||||||||
352 TCAGTACATCTACACAGAAGCCTCAGAGAGTCTTTGTGGTGTGAAACTGG 401

598 AAGTCAACAAATACCAGTACCTGCTGACAGGGCGCGTGTATATGAAGGCAAG 647
    ||||||||||||||||||||||||||||||||||||||||||||||||||
402 AGGTCAACAAATACCAGTATCTGATTACAGGCCGCGTGTACGAAGGGAAG 451

648 ATGTACACAGGACTGTGCAACTTTGTGGAGAGGTGGGACCACCTCACACT 697
    ||||||||||||||||||||||||||||||||||||||||||||||||||
452 GTTTACACTGGCCTGTGCAATTGGTATGAGAAATGGGACCGACTGACTCT 501

698 GTCCCAGCGCAAGGGCCTCAATTACCGCTACCACCTGGGTTGCAATTGCA 747
    ||||||||||||||||||||||||||||||||||||||||||||||||||
502 GTCCCAGCGTAAAGACTGAATCATCGTTATCATCTGGGCTGTGGATGCA 551
```

FIG. 5(C)

```
748 AGATCAAGTCCTGCTACTACTTGCCTTGTTTTTGTGACCTCCAAGAATGAG 797
    ||||||||||||||||||||||||||||||||||||||||||||||||||
552 AGATTCGGCCCTGCTACTATTTGCCCTGCTTTTGCCACCTCCAAGAATGAG 601

798 TGTCTCTGGACCGACATGCTCTCCAATTTTTGGGTACCCTGGCTATCAGTC 847
    ||||||||||||||||||||||||||||||||||||||||||||||||||
602 TGCATTTTGGACAGACATGCTCTCCAACTTCGGCCACTCAGGACACCAAGC 651

848 CAAACACTACGCCTGCATCCGGCAGAAGGGTGGCTACTGCAGCTGGTACC 897
    ||||||||||||||||||||||||||||||||||||||||||||||||||
652 GAAGCACTATGCCTGCATCCAGAGGGTGGAAGGTTACTGCAGCTGGTATA 701

898 GAGGATGGGCTCCCCCAGACAAGAGCATCAGCAACGCCACAGACCCCTGA 947
    ||||||||||||||||||||||||||||||||||||||||||||||||||
702 GAGGATGGGCGCCTCCAGATAAAACGATCATCAATGCCACAGATCCCTGA 751

948 AC...CCAGACCTTCCCCACCT..CACCTCCTTCCCATCCCGCCGAGCGT 992
    ||||||||||||||||||||||||||||||||||||||||||||||||||
752 GCACGCTGTACCTTCCTTATCTTCCCTCTCCTTACTTGTGGCTGATCTT 801

993 CC..CAGACACTAACTCTTCCC.....AGAT..TGAC...GAA..TAGT 1031
    ||||||||||||||||||||||||||||||||||||||||||||||||||
802 CCTTTGGACACTAACTCTTACCCGATCATGATGACAATGAAATTAGT 851

1032 ...TGT....CTT....AAT..TAGCACTGGGGACACTTAAAGTCTCTGCTG 1080
     ||||||||||||||||||||||||||||||||||||||||||||||||||
852 GCCTGTTTTTCTTGCAAATTCTAGCACTTCGAACCG..... 886
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FIG. 6(A)

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TIMP-1   bTIMP-1[a] ----MAPFAPMASGI LLLLWLTAPSRACTC VPPHPQTAFCNSDVV IRAKFVGTAEVNE-- -----TALYQRYEIK -MTKMFKGFSALRDA  78
         pTIMP-1[b] ----MSPFAPLASGI LLLLWLTAPSRACTC VPPHPQTAFCSSDLV IRAKFVGAPEFNQ-- -----TASYQRYEIK -MTKMFKGFNALGDA  78
         hTIMP-1[c] ----MAPFEPLASGI LLLLWLIAPSRACTC VPPHPQTAFCNSDLV IRAKFVGTPEVNQ-- -----TTLYQRYBIK -MTKMYKGFQALGDA  78
         rTIMP-1[d] ----MAPLAALASSM LLLLWLVAPSRACTC VPPHPQTAFCNSDLV IRAKFVGAPEVNH-- -----TTLYQRYEIK -TTKMFKGFDALGHA  78
         mTIMP-1[e] ---MMAPPASLASGI LLLLSLIASSKACSC APPHPQTAFCNSDLV IRAKFMGSPEINE-- -----TTLYQRYKIK -MTKMLKGFKAVGNA  79
         mTIMP-1[f] ---MMAPFASLASGI LLLLSLIASSKACSC APPHPQTAFCNSDLV IRAKFMGSPRINE-- -----TTLYQRYKIK MMTKMLKGFKAVGNA  80

TIMP-2   hTIMP-2[g] MGAAARTLR-LALGL LLLATLLRPADACSC SPVHPQQAFCNADVV IRAKAVSEKEVDSGN DIYGNPIKRIQYEIK -QIKMFKGPE-----  83
         mTIMP-2[h] MGAAARSLR-LAHGL LLLASLLRPADACSC SPVHPQQAFCNADVV IRAKAVSEKEVDSGN DIYGNPIKRIQYEIK -QIKMFKGPD-----  83
         bTIMP-2[i] MGAAARSLP-LAFCL LLLGTLLPRADACSC SPVHPQQAFCNADIV IRAKAVNKKEVDSGN DIYGNPIKRIQYEIK -QIKMFKGPD-----  83

TIMP-2   cTIMP-3[j] ---MTAWLGFLAVFL CSWSLRDLVAEACTC VPIHPQDAFCNSDIV IRAKVVGKKLMKDG- -----PFGTMRYTVK -QMKMYRGFQI---M  77
         mTIMP-3    ---MTPWLG-LVVLL SCWSLGHWGAEACTC SPSHPQDAFCNSDIV IRAKVVGKKLVKEG- -----PFGTLVYTIK -QMKMYRGFSK---M  76

         bTIMP-1[a] PDIRFIYTPAMESVC GYFHRSQNRSEEFLI AGQ-LSNGHLHITTC SFVAPWNSMSSAQRR GFT-KTYAAGCEECT VFPCSSIPCKLQSDT  166
         pTIMP-1[b] PDIRFIYTPAMESVC GYFHRSQNRSQEPLI AGQ-LWNGHLHITTC SFVAPWNSLSSAQRQ GFT-EIYAAGCEECT VPPCTSIPCKLQSDT  166
         hTIMP-1[c] ADIRFVYTPAMESVC GYFHRSHNRSEEPLI AGK-LQDGLLHITTC SFVAPWNSLSLAQRR GFT-KTYTVGCEECT VPPCLSIPCKLQSGT  166
         rTIMP-1[d] TDIRFVYTPAMESVC GYSHKSQNRSEEFLI AGQ-LRNGLLHITTC SFVVPWNSLSFSQRS GFT-KTYAAGCDMCT VPACASIPCHLESDT  166
         mTIMP-1[e] ADIRYAYTPVMESLC GYAHKSQNRSEEFLI TGR-LRNGNLHISAC SFLVPWRTLSPAQQR AFS-KTYSAGCGVCT VFPCLSIPCKLESDT  167
         mTIMP-1[f] ADIRYAYTPVMESLC GYAHKSQNRSEEPLI TGR-LRNGKFHINAC SFLVPWRTLSPAQQR VFSKKNYSAGCGVCT VFPCLSIPCKLESDT  169

         hTIMP-2[g] KDIEFIYTAPSSAVC GVSLDVG-GKKEYLI AGKAEGDGKMHITLC DFIVPWDTLSTTQKK SLN-HRYQMGC-ECK ITRCPMIPCYISSPD  170
         mTIMP-2[h] KDIEFIYTAPSSAVC GVSLDVG-GKKEYLI AGKAEGDGKMHITLC DFIVPWDTLSITQKK SLN-HRYQMGC-ECK ITRCPMIPCYISSPD  170
         rTIMP-2[i] QDIEFIYTAPAAAVC GVSLDIG-GKKEYLI AGKAEGNGNMHITLC DFIVPWDTLSATQKK SLN-HRYQMGC-ECK ITRCPMIPCYISSPD  170

         cTIMP-3[j] PHVQYIYTEASESLC GVKLEV--NKYQYLI TGR-VYEGKVYTGLC NNYEKWDRLTLSQRK GLN-HRYHLGC-GCK IRPCYYLPCFATSKN  162
         mTIMP-3    PHVQYIHTEASESLC GLKLEV--NKYQYLL TGR-VYEGKMYTGLC NFVERWDHLTLSQRK GLN-YRYHLGC-NCK IKSCYYLPCFVTSKN  161
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FIG. 6(B)

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bTIMP-1[a] HCLWTDQLLTGSDKG FQSRHLACLPREPGL CTWQSLRAQMA---- ----- 207
pTIMP-1[b] HCLWTDQLLTGSDKG FQSRHLACMPREPGM CTWQSLRPRVA---- ----- 207
hTIMP-1[c] HCLWTDQLLQGSEKG FQSRHLACLPREPGL CTWQSLRSQIA---- ----- 207
rTIMP-1[d] HCLWTDSSL-GSDKG FQSRHLACLPQEPGL CAWESLRPRKD---- ----- 206
mTIMP-1[e] HCLWTDQVLVGSED- YQSRHFACLPRNPGL CTWRSLGAR----- ----- 205
mTIMP-1[f] HCLWTDQVLVGSED- YQSRHFACLPRNLGL CTWRSLGAR----- ----- 207

hTIMP-2[g] ECLWMDWVTEKNING HQAKFFACIKRSDGS CAWYRGAAPPKQEFL DIEDP 220
mTIMP-2[h] ECLWMDWVTEKSING HQAKFFACIERSDGS CAWYRGAAPPKQEFL DIEDP 220
bTIMP-2[i] ECLWMDWVTEKNING HQAKFFACIKRSDGS CAWYRGAAPPKQEFL DIEDP 220

cTIMP-3[j] ECIWTDMLSNFGHSG HQAKHYACIQRVEGY CSWYRGWAPPDKTII NATDP 212
mTIMP-3  ECLWTDMLSNFGYPG YQSKHYACIRQKGGY CSWYRGWAPPDKSIS NATDP 211
```

b = BOVINE
p = PIG
h = HUMAN
r = RABBIT
m = MOUSE
c = CHICKEN

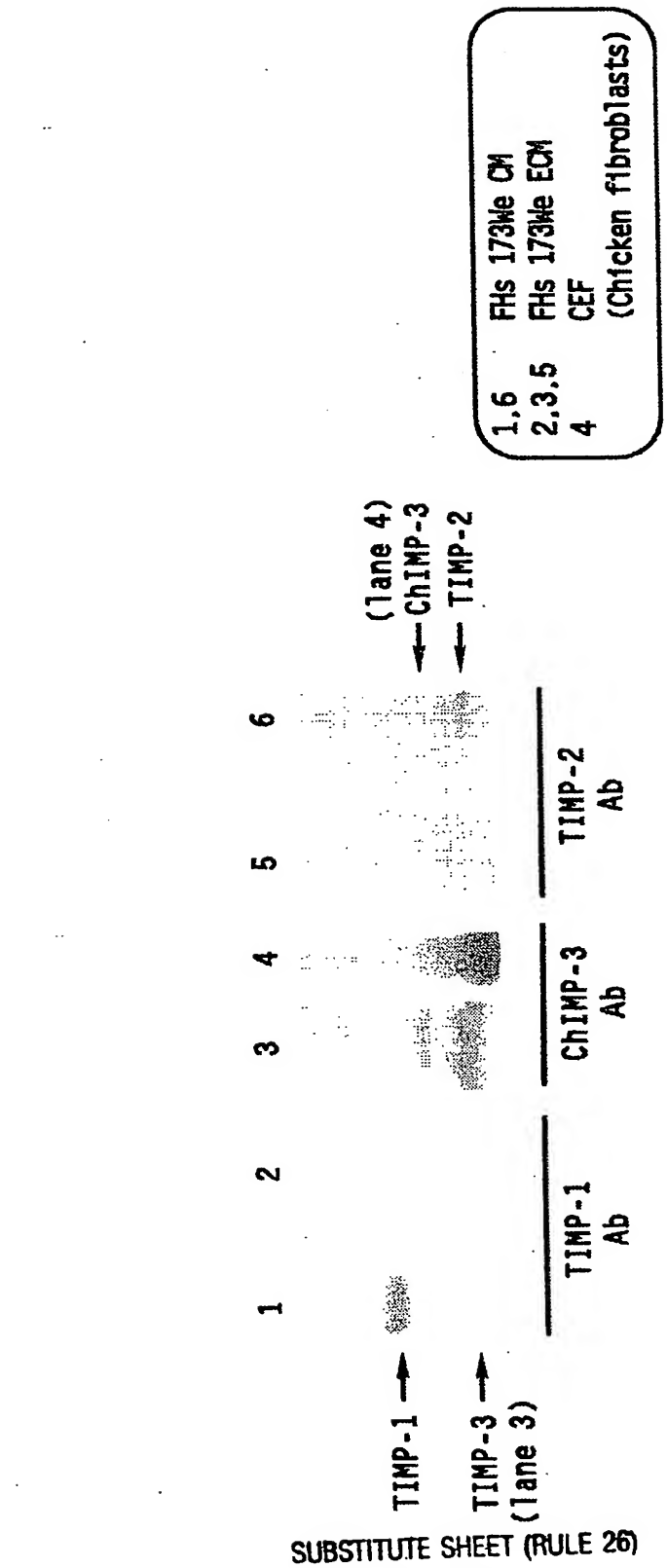
1,6 FHs 173We CM
2,3,5 FHs 173We ECM
4 CEF (Chicken fibroblasts)
(lane 4) ChIMP-3
TIMP-2
TIMP-1
TIMP-3 (lane 3)
1
2
3
4
5
6
TIMP-1 Ab
ChIMP-3 Ab
TIMP-2 Ab

FIG. 7

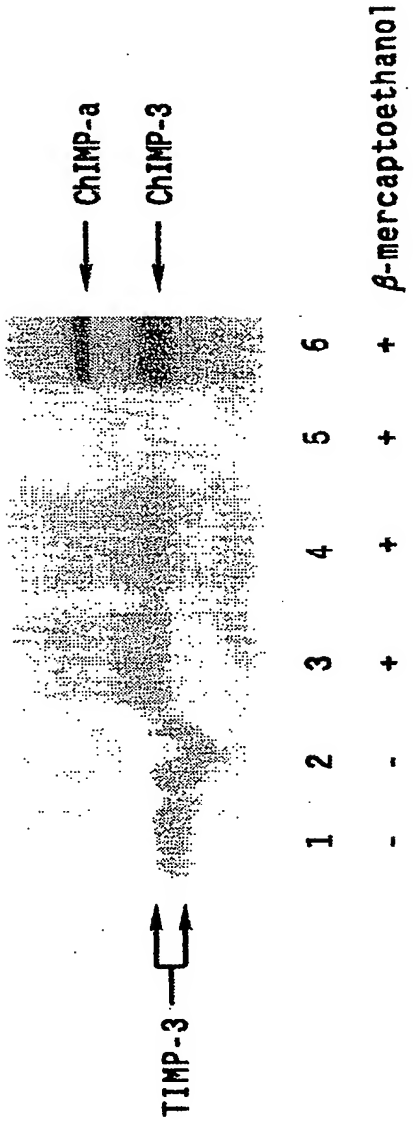

FIG. 8

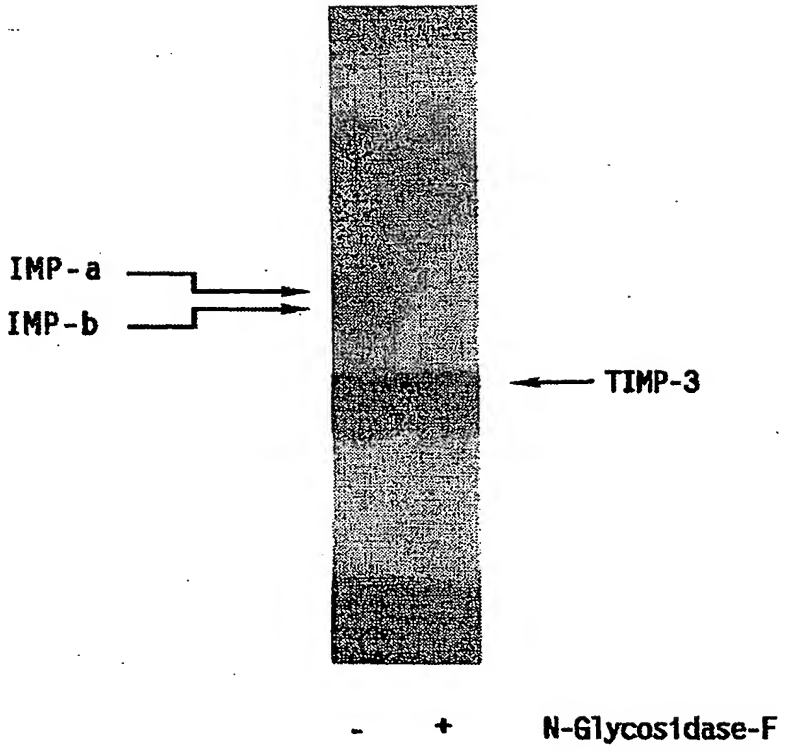

FIG. 9

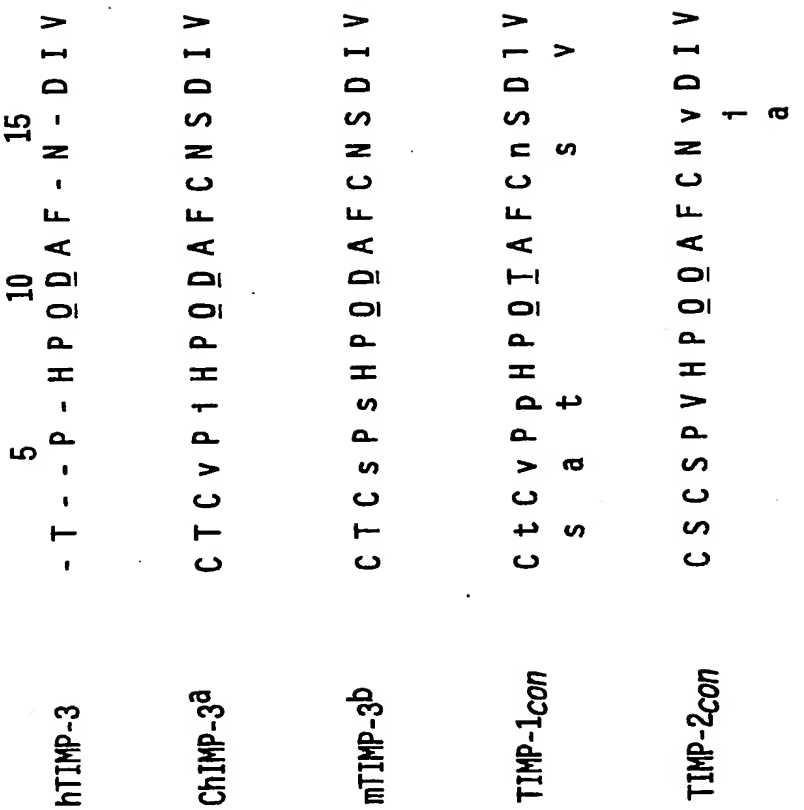
5 10 15
hTIMP-3 - T - - P - H P O D A F - N - D I V
ChIMP-3a C T C v P 1 H P O D A F C N S D I V
mTIMP-3b C T C s P s H P O D A F C N S D I V
TIMP-1con C t C v P P H P O I A F C n S D 1 V
s a t s v
TIMP-2con C S C S P V H P O O A F C N v D I V
1
a

FIG. 10

FIG. 11

```
G AAT TCC GGC GTG CTC CTG GGC AGC TGG AGC CTG GGG GAC TGG GGC GCC GAG
  N  S  G  V  L  L  G  S  W  S  L  G  D  W  G  A  E
   MATURE TIMP-3
GCG TGC ACA TGC TCG CCC AGC CAC CCC CAG GAC GCC TTC TGC AAC TCC GAC ATC
 A  C  T  C  S  P  S  H  P  Q  D  A  F  C  N  S  D  I

GTG ATC CGG GCC AAG GTG GTG GGG AAG AAG CTG GTA AAG GAG GGG CCC TTC GGC
 V  I  R  A  K  V  V  G  K  K  L  V  K  E  G  P  F  G

ACG CTG GTC TAC ACC ATC AAG CAG ATG AAG ATG TAC CGA GGC TTC ACC AAG ATG
 T  L  V  Y  T  I  K  Q  M  K  M  Y  R  G  F  T  K  M

CCC CAT GTG CAG TAC ATC CAC ACG GAA GCT TCC GAG AGT CTC TGT GGC CTT AAG
 P  H  V  Q  Y  I  H  T  E  A  S  E  S  L  C  G  L  K

CTG GAG GTC AAC AAG TAC CAG TAC CTG CTG ACA GGT CGC GTC TAT GAT GGC AAG
 L  E  V  N  K  Y  Q  Y  L  L  T  G  R  V  Y  D  G  K

ATG TAC ACG GGG CTG TGC AAC TTC GTG GAG AGG TGG GAC CAG CTC ACC CTC TCC
 M  Y  T  G  L  C  N  F  V  E  R  W  D  Q  L  T  L  S

CAG CGC AAG GGG CTG AAC TAT CGG TAT CAC CTG GGT TGT AAC TGC AAG ATC AAG
 Q  R  K  G  L  N  Y  R  Y  H  L  G  C  N  C  K  I  K

TCC TGC TAC TAC CTG CCT TGC TTT GTG ACT TCC AAG AAC GAG TGT CTC TGG ACC
 S  C  Y  Y  L  P  C  F  V  T  S  K  N  E  C  L  W  T

GAC ATG CTC TCC AAT TTC GGT TAC CCT GGC TAC CAG TCC AAA CAC TAC GCC TGC
 D  M  L  S  N  F  G  Y  P  G  Y  Q  S  K  H  Y  A  C

ATC CGG CAG AAG GGC GGC TAC TGC AGC TGG TAC CGA GGA TGG GCC CCC CCG GAT
 I  R  Q  K  G  G  Y  C  S  W  Y  R  G  W  A  P  P  D

AAA AGC ATC ATC AAT GCC ACA GAC CCC TGA GCG CCAGA CCCTG CCCCA CCTCA
 K  S  I  I  N  A  T  D  P

CTTCC CTCCC TTCCC GCTGA GCTTC CCTTG GACAC TAACT CTTCC CAGAT GATGA CAATG

AAATT AGTGC CTGTT TTCTT GCAAA TTTAG CACTT GGAAC ATTTA AAGAA AGGTC TATGC

TGTCA TATGG GGTTT ATTGG GAACT ATCCT CCTGG CCCCA CCCTG CCCCT TCTTT TTGGT

TTTGA CATCA TTCAT TTCCA CCTGG GAATT TCTGG TGCCA TGCCA GAAAG AATGA GGAAC

CTGTA TTCCT CTTCT TCGTG ATAAT ATAAT CTCTA TTTTT TTAGG AAAAC AAAAA TGAAA

AACTA CTCCA TTTGA GGATT GTAAT TCCCA CCCCT CTTGC TTCTT CCCCA CCTCA CCATC

TCCCA GACCC TCTTC CCTTT GCCCT TCTCC TCCAA TACAT AAAGG ACACA GACAA GGAAC

TTGCT GAAAG GCCAA CCATT TCAGG ATCAG TCAAA GGCAG CAAGC AGATA GACTC AAGGT

GTGTG AAAGA TGTTA TACAC CAGGA GCTGC CACTG CATGT CCCAA CCAGA CTGTG TCTGT

CTGTG TCTGC ATGTA AGAGT GAGGG AGGGA AGGAA GGAAC TACAA GAGAG TCGGA GATGA

TGCAG CACAC ACACA ATTCC CCCCG GAATT C
```

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09188

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :Please See Extra Sheet.
US CL :435/7.1, 23, 69.2; 530/350, 387.9; 536/23.5; 514/12
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.1, 23, 69.2; 530/350, 387.9; 536/23.5; 514/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, CAS, BIOSIS, MEDLINE, LIFESCI, EMBASE, BIOTECHDS, WPIDS
search terms: timp, gene# or sequence#, chimp, metalloproteinase, inhib?

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X ------ P, Y	CANCER RESEARCH, Volume 54, issued 15 April 1994, J.A. Uria et al., "Structure and Expression in Breast Tumors of Human TIMP-3, a New Member of the Metalloproteinase Inhibitor Family", pages 2091-2094, see entire document.	6-8 ----- 1-5, 9-15
P, X ------ P, Y	JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 269, No. 29, issued 22 July 1994, M. Wick et al., "A Novel Member of the Human Tissue Inhibitor of Metalloproteinases (*TIMP*) Gene Family Is Regulated During G_1 Progression, Mitogenic Stimulation, Differentiation, and Senescence", pages 18953-18960, see entire document.	6-8 ----- 1-5, 9-15

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
18 NOVEMBER 1994	12DEC 1994
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer REBECCA PROUTY Telephone No. (703) 308-0196

Form PCT/ISA/

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09188

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X ----- P, Y	FASEB JOURNAL, Volume 8, No. 4, issued 15 March 1994, N.S. Kishinani et al., "Interactions of TIMP-3 With ECM Components Are Influenced By Charged Polymers", page A47, abstract no. 271, see entire abstract.	1-5 ----- 6-15
P, X ----- P, Y	GENE, Volume 141, issued April 1994, S.M. Silbiger et al., "Cloning of cDNAs Encoding Human TIMP-3, a Novel Member of the Tissue Inhibitor of Metalloproteinase Family", pages 293-297, see entire document.	6-8 ---- 1-5, 9-15
P, X ----- P, Y	FASEB JOURNAL, Volume 8, No. 5, issued 18 March 1994, C.G. Wilde et al., "Identification of Human TIMP-3 by High-Throughput cDNA Cloning and Database Discovery", page A936, abstract no. 5423, see entire abstract.	6-8 ----- 1-5, 9-15
P, X ----- P, Y	DNA AND CELL BIOLOGY, Volume 13, No. 7, issued July 1994, C.G. Wilde et al., "Cloning and Characterization of Human Tissue Inhibitor of Metalloproteinases-3", pages 711-718, see entire document.	6-8 ----- 1-5, 9-15
P, X ----- P, Y	GENOMICS, Volume 19, issued January 1994, S.S. Apte et al., "Cloning of the cDNA Encoding Human Tissue Inhibitor of Metalloproteinases-3 (TIMP-3) and Mapping of the TIMP3 Gene to Chromosome 22, pages 86-90, see entire document.	6-7 ----- 1-5, 8-15
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, Volume 89, issued November 1992, T. Yang et al., "Role of the 21-kDa Protein TIMP-3 in Oncogenic Transformation of Cultured Chicken Embryo Fibroblasts", pages 10676-10680, see entire document.	1-15
Y	JOURNAL OF BIOLOGICAL CHEMISTRY, Volume 267, No. 24, issued 25 August 1992, N. Pavloff et al., "A New Inhibitor of Metalloproteinases From Chicken: ChIMP-3", pages 17231-17236, see entire document.	1-15
Y	WO, A, 90/11287 (STETLER-STEVENSON ET AL.) 04 October 1990, see entire document.	1-15
Y	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES USA, Volume 87, issued April 1990, T.C. Boone et al., "cDNA Cloning and Expression of a Metalloproteinase Inhibitor Related to Tissue Inhibitor of Metalloproteinases", pages 2800-2804, see particularly page 2801 and Figure 2.	1-15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09188

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GENE, Volume 114, issued May 1992, S.Shimizu et al., "Cloning and Sequencing of the cDNA Encoding a Mouse Tissue Inhibitor of Metalloproteinase-2", pages 291-292, see entire document.	1-15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US94/09188

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

C12P 21/02; C07K 14/81, 16/38; C12N 15/15; C12Q 1/37; A61K 38/57; G01N 33/68

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

Group I, claims 1-5 and 13-15, drawn to human TIMP-3 protein and compositions.

Group II, claims 6-8, drawn to human TIMP-3 DNA.

Group III, claims 9-12, drawn to antibodies and immunoassays for human TIMP-3.

The inventions listed as Groups do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: The proteins of Groups I and III and the DNA of Group II are structurally distinct compounds.